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TITLE: Production of Polyclonal Antibodies in Rabbits

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October 1995 Final, Phase II 1 Mar 93 - 31 Aug 95

Production of Polyclonal Antibodies in Rabbits DAMD17-92-C-2047

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2172-F

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Studies were carried out on the effects of repeated injections of bovine fetal serum acetylcholinesterase and horse serum butyrylcholinesterase in rabbits. Concentrations of these enzymes became signficantly elevated after one or two injections. The elevated levels persisted for many days. Several subsequent injections were given at intervals of approximately four months. In each case, these subsequent injections induced production of IgG specific to the heterologous enzyme. Also under this contract, polyclonal antibodies against various proteins, peptides, and antibody fragments were produced in rabbits. Finally, injectable microspheres containing butyrylcholinesterase were prepared and evaluated for rate of release and retention of activity *in vitro*. One formulation was tested in rabbits.

Polyclonal antibodies, butyrylcholinesterase, rabbits, acetylcholinesterase, microspheres, microcapsules, sustained release 102

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[signature] 10/2/95

PI - Signature Date

POLYCLONAL ANTIBODIES IN RABBITS

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

I. INTRODUCTION

A. The Nature of the Problem

The project was directed to the Medical Chemical Defense Research Program. Under the broad mission of providing medical countermeasures to chemical warfare agents, the work was relevant to develop the use of exogenous cholinesterase enzymes as a pretreatment to protect against organophosphorous chemical agents.

The threat of deadly exposure to chemical agents is still significant for American military personnel and the American public. The recent use of sarin by Japanese extremists in subway stations in Japan is witness to that. Amongst the most lethal chemical agents are organophosphorous (OP) compounds such as soman, sarin, and tabun. Poisoning from these agents can lead to rapid and severe dysfunction and even death from respiratory failure within five minutes **(Gilman, et al., 1990)**.

These deadly agents act by inhibiting enzymes (cholinesterases) crucial in neuronal control of many biological functions. The enzymes are widely distributed in the central and peripheral nervous systems and also in plasma, brain, muscle, and other tissues. The cholinesterases fall into two separate families: true cholinesterase, also known as acetylcholinesterase (AChE); and pseudocholinesterase, also known as butyrylcholinesterase (BChE). AChE hydrolyzes acetylcholine (ACh), a compound which serves as the neurohumoral agent in peripheral junction transmission. The function of AChE is to terminate the action of ACh at the junctions of the various cholinergic nerve endings with their effecter organs or postsynaptic sites. AChE is crucial for cholinergic neurotransmission and occurs at high concentrations in the immediate vicinity of nerve endings where it hydrolyzes ACh in less than a millisecond. The enzyme is closely associated with neuronel membranes **(Goodman and Gilman, 1990)**. Butyrylcholinesterase is present to a limited extent in neural elements and is also present in plasma, liver, pancreas, and other organs.

Compounds that inhibit cholinesterases are called anticholinesterases (anti-ChE). They cause accumulation of ACh at cholinergic receptor sites and this accumulation produces effects equivalent to excessive stimulation of these receptors throughout the peripheral and central nervous systems. In view of the essential nature of AChE, anti-ChE agents have received extensive attention as potential chemical-warfare nerve gases. Initial anti-ChE work prior to 1940 focussed on "reversible" agents, of which physostigmine is a well known example. Since then, several extremely toxic organophosphorous nerve gas OP's which "irreversibly" inactivate AChE, have been synthesized, including sarin, soman, and tabun. Other OP's are employed as agricultural insecticides, the foremost examples being parathion, malathion, and diazinon.

Because exposure to an OP agent causes extremely rapid and potentially lethal incapacitation, and because the threat of exposure remains serious, it is a high priority to provide United States military personnel with complete and safe protection from any such agent. The most effective mode of protection is a complete physical barrier, excluding any direct contact with agent through eyes, nose, mouth, or skin. While protective gear and masks are available, it takes time to "suit up" and it is difficult to carry out normal tasks wearing protective clothes and a gas mask. Obviously, this gear cannot be worn at all times.

If exposure to OP agent occurs without protection, prompt administration of atropine and pralidoximine (2-PAM) may be life saving. However, even if this treatment is successful, the individual is unlikely to be capable of executing battlefield functions for 18-24 hours or longer **(Taylor, 1990)**.

It has long been a goal to provide a pretreatment to individuals at risk for exposure to OP. A realistic target for duration of protection is 21-28 days. A minimum level of protection that has been suggested is the ability to inactivate two LD_{50}'s of soman in blood. The prophylactic material should not impair performance or cause any residual effects. Furthermore, any substance used in prophylaxis for exposure to chemical agents should be appropriate for repeated use without development of tolerance or analyphylaxis.

In fact, the drug pyridostigmine, a pyridine carbamate, is utilized for this purpose. The mechanism of action is reversible inhibition of AChE, which means that while present, the drug blocks some of the enzyme at the neuronal sites, protecting it from irreversible inactivation by OP.

Another approach to protection is to inactivate the OP molecules as they enter the blood. While OP's on surfaces of vehicles or impermeable clothing can be readily inactivated by strong sodium hydroxide solutions, this mode is hardly appropriate for blood. In general, small molecules that might inactivate the agent are nonspecific in action and unsuitable. Specificity of treatment is gained with larger molecules which can differentiate among potential targets. These are the characteristics of enzymes, immunoglobulins, and receptor molecules. The ideal OP scavenger in the blood would be highly specific for OP, it would have no side effects (acute or long term), and it would be effective after repeated doses.

A simple prophylactic approach, which appears to meet the criteria, is to provide the body with an excess of the agents' intended target: cholinesterase enzymes or fragments of such enzymes. While an increased concentration of AChE at neuronal junctions would be likely to have serious problems, increased blood levels of soluble ChE's such as butyrylcholinesterase or fetal bovine serum acetylcholinesterase would have little effect on normal functioning.

Our Phase II SBIR program has been in support of this latter approach to prophylactic protection against OP poisoning. We have carried out clearance studies and repeated injection studies of horse serum butyrylcholinesterase (E-BChE) and fetal bovine serum acetylcholinesterase (FBS-AChE) in rabbits. Additionally, we have collected polyclonal antibody to these enzymes, related synthetic peptides, and FAB fragments from monoclonal antibodies against FBS-AChE. Additionally, we prepared and tested formulations to extend the presence of E-BChE in blood.

B. Background

1. Prophylactic Use of Cholinesterases for Protection from OP Poisoning

Research at WRAIR and by others on the use of cholinesterases for protection from OP poisoning clearly demonstrates that animals can be protected against the toxic effects of organophosphorous agents by injections of cholinesterase enzymes such as fetal bovine serum acetylcholinesterase (FBS-AChE), horse serum butyrylcholinesterase (E-BChE), and human serum butyrylcholinesterase enzymes (H-BChE) **(Wolfe, et al., 1987; Caranto, et al., 1994; Doctor, et al., 1993; Ashani, et al., 1991; Raveh, et al., 1989; Broomfield, et al., 1991, and Maxwell, et al., 1992).**

For example, **Wolfe, et al. (1987)** dosed mice with FBS-AChE and found that the exogenous enzyme provided protection from VX and soman. Efficacy was determined by two factors: survival rate and ability to retain or rapidly regain baseline performance levels. **Raveh, et al. (1989)** reported studies in mice, showing protection from 8 LD_{50}'s of MEPQ with FBS-AChE. **Broomfield, et al. (1991)** worked with E-BChE and showed that Rhesus monkeys were protected from soman poisoning with exposure of two LD_{50}'s or even 3-4 LD_{50}'s. The ability of the monkeys to perform tasks at baseline level within eight hours of exposure was reported. Further work in mice, marmosets, and monkeys demonstrated the linear correlation between blood AChE levels and protection against challenge with organophosphorous agents (**Doctor, et al., 1991**).

In another study by **Ashani, et al.** (1991), H-BChE and H-AChE were shown to protect mice from multiple LD_{50}'s of soman. **Wolfe, et al.** (1992) used FBS-AChE and E-BChE as pretreatment drugs for the prevention of soman toxicity in Rhesus monkeys. Protection was shown for up to five LD_{50}'s of soman, and no significant performance deficits were observed. **Maxwell, et al. (1992)** also showed protection of Rhesus monkeys against soman with FSB-AChE. These results clearly demonstrate that exogenous cholinesterase enzymes are the most promising drugs available today for pretreatment against the toxic effects of organophosphorous chemical warfare agents.

The lack of availability of large amounts of enzyme was very successfully addressed recently by **Doctor, et al.** (1993) and by **Caranto, et al.** (1994). These investigators reported that the use of oximes in conjunction with exogenous cholinesterase enzymes improves the efficacy of the enzymes and the level of protection. In studies by **Doctor, et al.** (1993), monkeys were given enzyme injections then repeated doses of sarin and oxime. The oxime HI-6 was shown to be more effective than 2-PAM and other oximes. In further work by **Caranto, et al.** (1994), mice were treated with FBS-AChE and HI-6 then challenged with sarin. The results showed 57-fold reduction in the amount of enzyme required to counteract the effects of sarin. Thus, the use of oximes, such as HI-6, not only allows the use of exogenous enzymes as a prophylactic, but also **significantly reduces the dose** requirements of the enzyme to levels that make their use a viable and economic method for use against the toxic effects of organophosphorous agents.

These studies have demonstrated the following about the use of exogenous cholinesterase enzymes as pretreatment to protect against organophosphorous chemical agents.

1. Large parenteral doses of the enzyme can be administered safely to mice, rats, rabbits, and non-human primates with no or only transient effects on their ability to perform tasks. **Wolfe, et al.** (1987) showed that 11,000 units can be safely administered to mice.

2. The enzymes provide protection against organophosphorous chemical agents as determined by survival rate and ability to retain baseline performance levels (**Doctor, et al., 1991; Raveh, et al., 1989; and others**).

3. The level of protection can be improved with concomitant reduction in the enzyme dose by the use of oximes, such as HI-6 (**Doctor, et al., 1993; Caranto, et al., 1994**).

2. BIOTEK Phase I Work on Production of Polyclonal Antibodies to Cholinesterases and Related Peptides

In Phase I, BIOTEK prepared several antisera to cholinesterase and related peptides materials. The materials, supplied by Walter Reed Army Institute of Research (WRAIR), were:

1. Horse serum butyrylcholinesterase (E-BChE).
2. Bovine fetal serum acetylcholinesterase (FBS-AChE).
3. Seven "Cocktails" of peptides relevant to the active site of bovine fetal serum acetylcholinesterase. There were four types of peptides ranging from 35-43 aminoacids long, representing sequences from the native enzyme. Each cocktail contained three or four peptide types incorporated in liposomes.

The antisera were raised in SPF New Zealand White rabbits. Titers against the peptides were measured in the rabbits given peptide cocktails by ELISA (Enzyme Linked Immunosorbant

Assay) at BIOTEK. Plasma levels of the enzymes and antibodies to the enzymes were monitored throughout the study. The plasma enzyme and antibody levels were quantified at WRAIR.

These studies were the precursors to the Phase II repeated injection studies, which are described in this report.

C. Purpose of The Phase II Work

In Phase II, BIOTEK pursued the development of an enzyme-based prophylactic method for prevention of OP poisoning. The company carried out long term repeated ChE dosing studies in rabbits and also production of rabbit polyclonal antibodies for related studies. The purpose was to demonstrate that elevated blood cholinesterase levels could be attained and maintained by the injection of exogenous enzyme.

The first objective of Phase II was to follow the long term effect on the immune system of the rabbit after repeated injections of **purified horse serum butyrylcholinesterase and fetal bovine serum acetylcholinesterase**, and also to determine the half-life of butyrylcholinesterase in circulation when injected intravenously and also intramuscularly in rabbits. **This objective was completed.**

The second objective was to produce antibodies against **natural and synthetic peptides in the rabbit**. The immunogens used in the preparation of the antibodies were supplied by WRAIR. The antisera were submitted to WRAIR for use in the development of assay procedures, localization of cholinesterase activity, and understanding the mechanisms of action in various tissues. **This objective was completed.**

The third objective was to produce antibodies against **Fab fragments** from monoclonal antibodies against fetal bovine serum acetylcholinesterase. A population of anti-idiotypic antibodies was obtained after a series of injections of each Fab fragment into rabbits. **This objective was completed.**

The fourth objective was to prepare and evaluate **injectable butyrylcholinesterase containing microcapsule formulations** which would release active enzyme for one to four weeks. This work would test the hypothesis that continuous release of the enzyme would result in a lower generation of anti-BChE antibodies in rabbits. **This objective was completed.**

D. Methods of Approach

1. Preparation of Polyclonal Antisera in Rabbits

Proteins, peptides, carbohydrates, nucleic acids, lipids, and many other naturally occurring or synthetic compounds can act as successful immunogens. An immunogen must first have an epitope that can be recognized by the cell surface antibody found on B cells. Second, it must have at least one site that can be recognized simultaneously by a class II protein and by T-cell receptors. Finally the compound must be biodegradable. In general, molecules less than 3,000-5,000 Daltons (i.e. haptens) are not good immunogens. This is overcome by physical coupling of the hapten to larger immunogenic molecules or carriers. Haptens are usually coupled to soluble carriers such as bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). Synthetic peptides have been used as immunogens (haptens) for elucidating the properties of antibody response (**Sela, 1960; Arnon, et al., 1971**). This permits the production of site-specific antibodies useful in functional and clinical studies. In this program, proteins, peptides and fragments of large proteins (monoclonal antibodies) have been used as immunogens.

The physical form of the antigen plays an important role in eliciting antibody response. Antigens prepared in the form of small particles or aggregates are better immunogens than soluble proteins. Better response is obtained when soluble proteins are aggregated by self polymerization, coupled chemically to large matrices such as agarose or bound to large carrier proteins. In this program some of the peptide haptens were incorporated in liposomes to present them as aggregates. Other peptides were conjugated to larger protein carriers.

Two mechanisms have been proposed to explain how insolubility enhances the immune response of an animal to an antigen (**Osebold, 1982**). The first involves the formation of a depot or reservoir which protects the antigen from rapid degradation, thus prolonging its exposure to the hosts's immune system. In the second mechanism, adjuvants enhance immune response by attracting immune system cells and non-specifically activating them.

A commonly used adjuvant for research work is Freund's adjuvant. Freund's adjuvant is a water-in-oil emulsion prepared with nonmetabolizable paraffin oil. If the mixture contains killed M. tuberculosis, it is referred to as complete Freund's adjuvant (CFA). Without the bacteria it is known as incomplete Freund's adjuvant (IFA). Freund's is one of the best adjuvants for stimulating strong and prolonged responses. The principal disadvantage of Freund's adjuvant is that is can invoke very aggressive and persistent granulomas. It is not used for injections of humans for this reason, and the possible side effects should be monitored carefully during antibody production in animals. To avoid the majority of the side effects, only the primary injection is given in CFA, while all boosts are administered in IFA. These adjuvants were used with conjugated peptides to raise polyclonal antibodies under this Phase II program.

Rabbits represent a good choice for the routine production of polyclonal sera. They were chosen for use in this project because they are easy to keep and handle, they can be safely and repeatedly bled, and the antibodies they produce are well characterized and easily purified. With careful management, at least 500 ml of serum can be obtained from one rabbit through the course of an immunization regime. Most laboratory rabbits are outbred and relatively little is known of the genetics of the immune response in this species, but because they are outbred, they have a wider range of class II proteins or other immune response proteins than inbred animals.

2. Repeated Injection of Cholinesterases in Rabbits

Single injections of cholinesterase are effective as a prophylactic protection against OP, as discussed previously. For this treatment to be feasible for military use, many further issues must be addressed. It is important to understand the pharmacokinetics of such injections, the influence of route of injection on blood levels, and the effects of repeated injections of the enzyme. It was also of interest to learn the extent of antibody development against the enzymes and the efficacy of repeat injections given after the circulating antibody level had returned to baseline. Further, the systemic effects of repeated doses were unknown.

Studies to address these issues were carried out under this program. Two types of enzymes were chosen: horse serum butyrylcholinesterase and bovine serum acetylcholinesterase. These choices were made based on availability, as WRAIR was able to provide these materials, and on the familiarity with these enzymes.

The pharmacokinetic studies were carried out studying two routes of injection: I.M. and I.V. These were chosen because they would be the ways in which the prophylactic doses might be given to military personnel in the field.

The repeat injection protocol chosen was based on the premise that individuals need prophylactic protection from OP for one to four weeks. Several injections of the enzyme were given over a two week period. The blood was monitored for enzyme levels and for antibody against the enzyme given. When the antibody level returned to baseline, the enzyme was given again, mimicking a second period of prophylaxis. After four or five series of injections, cross reactivity with another cholinesterase was tested. Although there is extensive sequence homology among the cholinesterases, it might be possible to overcome some immune reactivity by alternating enzymes. Necropsies were planned to evaluate the long-term effects of the repeat injections.

3. Microsphere and Microcapsule E-BChE Delivery Systems

Until the present work, studies of administration of prophylactic dosing of cholinesterases had been carried out by I.M. administration of the enzyme. It was of interest to learn if a prolonged effect could be achieved by formulating the enzyme in such a way that it would be released over a period of 1 to 4 weeks into the blood from microspheres or microcapsules injected under the skin. In this way the number of injections needed for sustained protection would be reduced. It was also of interest to see if this method of delivery would increase tolerance to the increased enzyme levels.

The preparation and testing of cholinesterase formulations requires substantial amounts of enzyme. The most readily available material for such a purpose is horse serum butyrylcholinesterase (E-BChE). This is the same enzyme used in many of the other studies under this contract. Of primary concern is that the structure and activity of this enzyme are preserved during the formulation process. The most commonly utilized materials for prolonged delivery protein formulations are the lactide-co-glycolide polymers. Several decades of experience have shown, however, that it is difficult to retain protein activity in such systems. A probable causative agent is the organic solvent used to process such polymers.

Prior to the initiation of Phase II, BIOTEK had begun development of an entirely different protein binder system: mineral microspheres prepared without organic solvent. The incorporation of E-BChE in such microspheres was considered an appealing route to the development of long-acting cholinesterase formulations. The award to BIOTEK of a Phase II NIH SBIR contract to continue studies of the mineral microspheres provided the opportunity for a synergistic study of the incorporation of proteins including E-BChE in such microspheres.

E. Summary of Results

The project achievements are described fully in the following report sections. **Section Two** describes the rationale and methodology for seven in vivo studies. **Section Three** describes the rationale and methodology for the preparation and in vitro evaluation of E-BChE microspheres and microcapsules. In **Section Four**, the results of all studies are detailed and discussed. Our own evaluation of the program achievements and implications for future work are given in **Section Five**.

The Phase II program accomplishments are summarized below:

1) Immunization of rabbits with peptides incorporated in liposomes - fourteen peptides were used, with two rabbits used per peptide;
2) Preparation of polyclonal antibodies to thirteen peptide conjugates - three rabbits were used per peptide;
3) Preparation of polyclonal antibodies to two different Fab fragments from monoclonal antibodies against butyrylcholinesterase - three rabbits were used per fragment;
4) Determination of clearance of horse serum butyrylcholinesterase given to rabbits by two routes of injection: I.M. and I.V.;

5) Demonstration of long-term effects of repeated injections of horse serum butyryl-cholinesterase in rabbits, followed by injections of fetal bovine serum acetyl-cholinesterase (a 23-month study in five rabbits);
6) Demonstration of long-term effects of repeated injections of fetal bovine serum acetyl-cholinesterase (FBS-AChE) in rabbits followed by injection of horse serum butyryl-cholinesterase (E-BChE) (a 21-month study in five rabbits);
7) Development of a method of preparing E-BChE mineral microspheres;
8) Comparison of the effects of repeated injections of E-BChE microspheres vs E-BChE solutions in six rabbits (a 43-day study);

II. IN VIVO METHODS

A. General Methods

1. Rabbit Care

Production of rabbit polyclonal antisera and repeated injection studies in rabbits were performed at BIOTEK's Animal Research Facility in Woburn, Massachusetts. The facility houses only **Specific Pathogen Free** (SPF) rabbits and **Viral Antibody Free** (VAF) rodents in order to protect the health and well being of the animals. This also reduces the potential for animal loss due to disease and increases the potential for a higher yield of antisera, sometimes with higher titer. All procedures performed on the rabbits followed the guidelines established by WRAIR, the National Institutes of Health (NIH), and the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Resource Council.

Upon delivery to receiving, all rabbits were immediately brought to the animal facility where they were kept under observation for a one to two week period. Each animal was examined individually and received an I.D., tattoo or other appropriate permanent identification. Animals showing ill effects during observation were treated with appropriate medications or disposed of, when necessary. All animals were observed seven days a week to ascertain health status. Animals which were moribund or found dead were necropsied. All rabbits were housed separately.

To obtain good antibody responses, healthy and well-cared-for animals are essential. Upon arrival, all animals were monitored for clinical signs of disease. Routine surveillance for respiratory Pasteurella multocida infection and fecal analysis for coccidia oocysts was performed on 10% of the rabbits when they were received. The test animals were selected at random. In addition, periodic surveillance for the same pathogens was made on different animals.

Routine examination was made of the clinical condition of all animals arriving at the facility. For rabbits thorough examination was made of the eyes, ears, nose, teeth, coat, feet, and stools. Microscopic examination is made for ectoparasites using skin scraping or debris from ears if mites are suspected and for endoparasites or coccidia oocytes using fecal flotation methods if diarrhea was present.

All animals were visually examined daily and a record was kept of any observed abnormalities which were immediately reported to the veterinarian, Dr. James G. Fox. Treatment of animals was performed after consultation with the veterinarian. An index card was placed on the cage of any sick or injured animal containing the following information: project code number, animal number, species, strain, sex, symptoms, date, diagnosis, medication to be given, treatments, spaces for technician to initial and date after each treatment. The project code number insured confidentiality.

2. Blood Sampling

a. Bleeding Techniques

The arteries of the rabbit's ears are readily accessible and are suitable for collecting 10 ml for test bleeds of up to 9 ml per kg rabbit weight for production bleeds. The rabbit was placed in a restrainer and blood was drawn from the central auricular artery. To minimize stress and help bleeding, the rabbit was given a tranquilizer (acepromazine, 1.0 mg/kg S.C.) for vasodilation. Test bleeds were drawn using a 23 gauge Infusion set and a 10 ml syringe and collected into a glass tube. Larger amounts of blood were collected using a 20 gauge infusion set attached to a vacuum apparatus for collection into a glass tube.

b. Collection Volume and Frequency

No more than 15-20% of the rabbit's total blood volume was collected at any one time unless the bleeding procedure was terminal. Smaller volumes were obtained at more frequent intervals as long as no more than 15-20% of the total blood volume of the rabbit was taken in any two week period. The maximum blood volume withdrawn from rabbits was 6-9 ml/kg rabbit weight. For example, for a 4 kg rabbit, the maximum withdrawal was 24 to 36 ml.

c. Isolation of Anti-Serum

The blood sample was allowed to clot at room temperature and then refrigerated overnight at 4°C. The following day the serum was separated from the red blood cells by centrifugation at 2,500 rpm for 15 minutes. The sera were placed in labeled vials and frozen at -20°C until shipment.

d. Preparation of Blood for Enzyme Assays

For test bleeds, 250 μl of blood was collected in a Natelson 250 μl heparinized tube and diluted in 2.25 ml of cold distilled water, giving a 1/10 dilution. The labeled blood samples were immediately frozen at -20°C until shipment in insulated styrofoam boxes with ice packs.

3. Injection Techniques

I.V. injections were given in the marginal ear vein using a 1 ml syringe attached to a 25 gauge 5/8" needle. I.M. injections were given in the thigh muscle using a 1 or 3 ml syringe attached to a 23 gauge 1" needle. S.C. injections were given in the lower back area near hind legs using a 1 or 3 ml syringe attached to a 23 gauge 1" needle.

4. Exsanguination and Euthanasia

For exsanguinations, as much blood was drawn from the ear artery as possible, and then the rabbit was euthanized. For euthanasia, 1.0 ml of Somlethol was given I.V. in the marginal ear vein.

B. Butyrylcholinesterase Clearance Study

1. Study Design

The objective of the study was to determine the half-life of purified horse serum butyrylcholinesterase in the circulation of rabbits, and to compare the half-life of the enzyme obtained when it was given intravenously with that obtained following intramuscular administration. Four rabbits were used for the study: two (Nos. 101 and 102) for measuring intravenous clearance and two (Nos. 103 and 104) for measuring intramuscular clearance. Test bleeds were obtained before injection and at regular intervals on each rabbit after injection. The blood was analyzed by Ms. Mary K. Gentry at WRAIR for cholinesterase activity. The results were utilized at BIOTEK to calculate the clearance of the exogenous enzyme from the rabbit blood.

2. Injection and Blood Collection Schedule

The study started on June 14, 1993 and ended August 13, 1993. A pre-dose test bleed was made. Each dose was given either I.M. or I.V. as a single bolus containing 2,200 U of E-BChE. After the doses were administrated, test bleeds were made at 1, 2, 4, 7, 24, 72, 96, and 168 hours. The blood was collected and prepared for enzyme assay, as described in the General Methods Section and shipped frozen to WRAIR.

C. Repeated Long-Term Butyrylcholinesterase Injections and Cross-Over to Acetylcholinesterase Injections

1. Study Design

The objective of the long term repeated butyrylcholinesterase injection study was to determine the ability to repeatedly raise blood cholinesterase levels in rabbits and also to follow the effect on the immune system of repeated I.M. injections. Purified horse serum butyrylcholinesterase (E-BChE) was utilized as the exogenous enzyme because it was readily available, because it had been used in earlier cited studies, and because BChE from many species have a high degree of homology (**Gentry and Doctor, 1991**). Five rabbits were used in the study (Nos. 96-100). A total of ten doses of E-BChE were given I.M. Four injections were given within the first eleven days of the study to observe the ability to sustain high blood enzyme levels. Other series of injections were given when blood anti-BChE levels had dropped to baseline. The first of these subsequent series was approximately three months after the first injection, when two injections were given three days apart. On two other occasions at intervals of about 4-5 months, two injections were given one day apart.

After these rabbits had been exposed to E-BChE for over twenty months, it was of interest to measure the level of cholinesterase activity that could be achieved by injections of FBS-AChE. The potential anti-AChE antibody response was also of interest. Thus, three cross-over injections were made, given I.M. approximately five months after the last E-BChE injection. Approximately two months after the first FBS-AChE I.M. injection, a single I.V. injection of FBS AChE was given. An additional naive rabbit was also added to the I.V. injection series.

Throughout the study, test bleeds were collected and prepared for enzyme assay as described in the General Methods Section. The samples were sent to WRAIR for analysis of enzyme activity and anti-E-BChE antibody concentration.

2. Injection and Blood Collection

The study was started on June 21, 1993 (day 1) and consisted of a pre-bleed and an initial immunization (No. 1) given intramuscularly in the rabbit thigh muscle. Sequential injections were alternated between the right and left thigh muscles. Additional immunizations (Nos. 2 through 4) were given on days 4, 8, and 11, and were followed by test bleeds on days 5, 9, and 12. From day 12, test bleeds were collected every two weeks until the second round of injections. The second round of injections (Nos. 5 and 6) were given beginning on October 25, 1993 on days 127 and 130. Test bleeds were made the day after injection, one week after injection, and every two weeks thereafter. The third round of injections (Nos. 7 and 8) were given on March 21 and March 22, 1994 on days 274 and 275. Test bleeds were made on days 275, 276, 277, 281, 289, and then every two weeks thereafter. The fourth set of injections (Nos. 9 and 10) were given on September 19, 1994 and September 20, 1994 and test bleeds were days 457, 458, 459, 463, 470, and then every two weeks thereafter.

The study with these five rabbits was extended to a "cross-over" study. The rabbits were given intramuscular injections of FBS-AChE on days 617, 620, and 624. Test bleeds were taken on days 618, 621, 625, 631, and then every two weeks. The five rabbits plus one naive rabbit were given an I.V. injection of FBS-AChE on day 694 with test bleeds taken at two and five hours after injection on day 694. Test bleeds were also taken on days 696, 698, and 701. The study ended with exsanguination of the rabbits on day 703. All animals were sent to the Massachusetts Institute of Technology Department of Comparative Medicine for necropsy. Following consultation with Ms. Mary K. Gentry (who in turn consulted with the pathologist at WRAIR), the following tissues were collected and sent to Dr. Frank McConnell, a veterinary pathologist, for examination. The tissues selected by the WRAIR pathologist were: heart, liver, spleen, kidney, urinary bladder, and lymph nodes. Necropsy and histopathology reports are included in Appendix A. Throughout the study, blood was collected and prepared for enzyme assay as described in the General Methods section. A full schedule of injections, boost injections, and blood drawing is shown in **Table 1**.

D. Repeated Long-Term Acetylcholinesterase Injections and Cross-Over to Butyrylcholinesterase Injections

1. Study Design

The objective of this long term repeated dose study was to determine the ability to repeatedly raise blood AChE levels in rabbits and to follow antibody response to these repeated injections. Five rabbits (Nos. 105-109) were used in the study. The rabbits received seven I.M. injections of fetal bovine acetylcholinesterase (FBS-AChE) over a period of 19 months. Four injections were given within the first eleven days of the study. At intervals of about 5 months the rabbits were given injections of FBS-AChE on two sequential days. One more injection was given after approximately an eleven month interval. The study with these five rabbits was extended to a "cross-over" study with a single I.M. dose of E-BChE.

2. Injection and Blood Collection

The first round of injections (Nos. 1 through 4) was given beginning on October 25, 1993, on days 1, 4, 8, and 11. Test bleeds were taken on days 1, 2, 5, 9, and 12, with biweekly bleeds from then until the next round of injections. The second round of FBS-AChE injections (Nos. 5 and 6) was given on March 21, 1994 and March 22, 1994 on days 148 and 149. Test bleeds were taken on days 149, 150, 151, 155, 163, and every two weeks thereafter up to October 31, 1994. On February 27, 1995 (day 491), these rabbits received FBS-AChE injection No. 7. Test bleeds were taken on days 492, 493, 495, 498, 505, until day 597.

The study with these five rabbits was extended to a "cross-over" study. The rabbits were injected I.M. with E-BChE on day 597. Test bleeds were taken on days 597, 598, 599, 626, and the rabbits were euthanized on day 631 (July 17, 1995). All animals were sent to the Massachusetts Institute of Technology Department of Comparative Medicine for necropsy. Microscopic evaluation of selected tissues was provided by Dr. Frank McConnell, a veterinary pathologist. Necropsy and histopathology reports are included in Appendix A. The schedule of injections, doses, and blood drawing for these rabbits is shown in **Table 2**. Throughout the study, the blood was collected and prepared for enzyme and antibody assays, as described in the General Methods section.

E. Preparation of Polyclonal Antibodies Against Natural and Synthetic Peptides

1. Study One - Peptide Liposomes

a. Study Design

The purpose of the study was to obtain polyclonal antibodies against a series of peptides relevant to AChE and other proteins. The peptide derivations, the four letter codes used to identify them, and the animals injected are shown in Table 3. These peptides were incorporated in liposomes at WRAIR. Young New Zealand White rabbits were used. Each peptide was given to two rabbits in an alum (aluminum hydroxide gel) suspension. After the primary immunization, two boost immunizations were given at four and eight weeks (except to the rabbits which had received SCD4, which were exsanguinated at week 6). The remaining rabbits were exsanguinated and/or euthanized at week ten.

b. Immunization and Blood Collection Schedule

Pre-immune bleeds were taken just prior to the primary immunization. Pre-immune sera were shipped to Dr. Mark Carter at WRAIR on June 2 and 3, 1993. From each peptide vial 1.0 ml was withdrawn and emulsified with 1.2 of alum just prior to the primary immunization and boost. Each peptide suspension was divided between two rabbits, each receiving 1.0 ml, given I.M. and S.C. divided equally in four locations. Two S.C. injections were given in the lower back and I.M. injections were given in thigh muscles. On June 1, 1993 (week 0), half of the rabbits were given the (primary) immunization with peptides XNAT, XRAD, XCNB, SCD4, CD41, CD42, and CD43. The remaining rabbits were given the primary immunization the following day, on June 2, 1993 with peptides FLIP, XN8, NTR1, NTR2, NTR3, NTR4, and NTR5.

After four weeks, the first boost immunizations were given on June 29 and 30, 1993. The first test bleeds were taken at week 5, on July 6 and 7, 1993, and the sera were shipped to Dr. Carter on July 7 and 8, 1993. Following the results of the titer determination by Dr. Carter, all rabbits received a second boost at week 8 on July 27 and 28, 1993, except the SCD4 peptide rabbits, which were exsanguinated at week 6 on July 13, 1993. The remaining 26 rabbits received their second test bleed at week 9, on August 2 and 3, 1993. The sera were shipped to Dr. Mark Carter on August 3 and 4, 1993.

The study terminated on week 10. Following the results of the titer analysis by Dr. Carter, all rabbits from peptide groups CD41, CD43, FLIP, XN8, NTR1, NTR2, NTR3, NTR4, and one rabbit from group CD42 were exsanguinated on August 10 and 11, 1993. Rabbit groups XNAT, XRDA, CD43, NTR5, and one rabbit from group CD42 were euthanized on August 10 and 11, 1993 and the sera were shipped on August 11 and 12, 1993. The schedule of injections and blood drawing is shown in Table 4. All animals were healthy up to the termination of study.

2. Study Two - Peptide Conjugates

a. Study Design

The second study was initiated in order to obtain a greater yield of polyclonal antibodies against some of the peptides previously used in liposome form and to obtain polyclonal antibodies against other peptides. Since the antibody response to the peptide liposomal preparations was weak in the first study, in this study, the peptides were conjugated to proteins. The peptide deriva tions, the four letter codes used to identify them, and the animals injected are shown in **Table 3**. Four of the rabbits had been used in earlier studies of E-BChE clearance, and these are marked with an asterisk. A series of peptide conjugates were received from WRAIR. The procedure for polyclonal production from these materials was to inoculate groups of three young adult New Zealand White rabbits with each material, using Freund's complete adjuvant for the primary immunization, then Freund's incomplete adjuvant for subsequent boost injections.

b. Immunization and Blood Collection Schedule

Immediately before the primary injection, 200 μl of Freund's Complete Adjuvant was added to 200 μl of conjugated peptide preparation and the mixture was emulsified. Each animal received 50 μl I.M. in one thigh muscle and 50 μl S.C. in the lower back just anterior to the pelvis. These immunizations were performed on December 14 and 15, 1993. For the booster immunizations, the same procedure was followed, except that Freund's Incomplete Adjuvant was used and the I.M. injection was in the alternate thigh. Boosters were given at week 4 on January 11 and 12, 1994 and at week 8 on February 8 and 9, 1994. Test bleeds were taken at week 5, January 18 and 19, 1994 and the rabbits were exsanguinated at week 9, on February 16, 17, and 18, 1994. All procedures were as described in the General Methods Section. Sera was collected and stored frozen at -20°C or colder until shipment by Federal Express to WRAIR. The schedule for these animals is shown in **Table 5**.

F. Induction of Anti-Idiotypic Antibodies to Fab Fragments from Anti-FBS-AChE Monoclonal Antibodies

1. Study Design and Material Received from WRAIR

The purpose of the study was to produce polyclonal antibodies which would include a population of antiidiotypic antibodies to Fab fragments (E4-13D8 and E5-25B1) of certain monoclonal antibodies against bovine serum acetylcholinesterase. These were provided by WRAIR.

2. Immunization and Blood Collection Schedule

Pre-bleeds were taken prior to primary immunization. Three young adult New Zealand White rabbits were given primary immunizations with each preparation on Week 0 (October 19, 1994). Each rabbit was injected I.M. in thighs in two sites. Rabbit Nos. 401-403 received 200 μl of E4-13D8 (0.46 mg/ml) and Rabbit Nos. 404-406 each received 200 μl of E5-25B1 (0.44 mg/ml). At Week 4, (November 16, 1994), the first boost immunization was given. The E5-25B1 group received 200 μl (0.44 mg/ml) I.M. in two sites and the E4-13D8 group received 100 μl (0.46 mg/ml) I.M. in one site. At Week 5 (November 22, 1994) test bleeds for sera were taken from both groups.

On November 22, 2 ml of E4-13D8 (0.411 mg/ml) was received. At Week 8 (December 14, 1994), the second boost immunization was given. The E5-25B1 group received 200 μl (0.44 mg/ml) I.M. in two sites. The E4-13D8 group received 200 μl (0.411 mg/ml) I.M. in two sites. At Week 9 (December 21, 1994) test bleeds for sera were taken from both groups. The rabbits were exsanguinated at week 11 (January 4, 1995). The schedule of injection and blood drawing for these rabbits is shown in **Table 6**.

G. Effect of Repeated Injections of E-BChE Microspheres

1. Study Design

This study was modeled after a Phase I study in which rabbits had been given five doses of 400 U each (2,000 U total) of E-BChE solution over a period of 15 days. The E-BChE used in this study was provided by Ms. Mary K. Gentry. The intent was to mimic the previous study, except that three doses (given weekly on days 1, 8, and 15) in the form of microspheres or solution containing about 666 U per dose would be given to achieve the 2,000 U total. A smaller number of doses was selected because it was expected that the microspheres would extend the duration of release of BChE. The E-BChE used in this new study was from Sigma Chemical Co. (St. Louis, MO), and the values of potency given above for the microspheres and solutions refer to the potency reported by Sigma.

2. Immunization and Blood Collection Schedule

Three New Zealand White rabbits (Nos. 440, 441, & 442) received repeated injections of E-BChE microspheres, a blend of 72MSG2, 3, and 5, which had an activity of 3 U/mg (referenced to the activity reported by Sigma). Syringes containing 226 mg (669 U) of E-BChE microspheres were prepared on the day of use. Within 10 minutes of injection, 0.6-1.0 ml of suspending medium was drawn into the syringe, and air bubbles were expelled. The syringe contents were injected into the thigh muscle. It was observed that there was some residual in each syringe, thus the actual dose was slightly less than the total amount weighed into the syringe. Injections were given intramuscularly into the thigh on days 1, 8, and 15. A fresh suspension was made at each injection time.

Three New Zealand White rabbits (Nos. 437, 438, & 439) received repeated injections of E-BChE solution. The contents of a vial labeled as containing 5,600 U of E-BChE (Sigma C1057) were suspended in 2 ml of 25% glycerol in sterile water for injection. This solution was stored at -20°C until use. Within 15 minutes of use, an aliquot of this was diluted to 1,288 U/ml and 0.5 ml (644 U) was injected intramuscularly into the thigh of each rabbit on days 1, 8, and 15.

Blood samples were drawn from each group of rabbits before the first injection on day 1 and then at 1, 4, and 8 hours. Blood samples were then drawn on days 2, 3, 4, and 5. After the second injection on day 8, blood samples were drawn on days 9 and 12, and after the third injection on day 15, blood samples were drawn on days 16, 19, 22, 26, 29, and 43. The blood was collected and prepared for enzyme assay, as described in the General Methods Section. The labeled samples were stored at -20°C until shipment to WRAIR to Ms. Mary K. Gentry for analysis of BChE levels. The schedule of injections and test bleeds is shown in **Table 7**.

III. METHODS OF PREPARATION AND *IN VITRO* EVALUATION OF BChE MICROSPHERES AND MICROCAPSULES

A. Materials

1. Calcium Sulfate

Calcium sulfate ($CaSO_4$) hemi-hydrate was purchased from Sigma Chemical Company (Sigma Catalog No. C7411). It was dried at 130°C for four hours and stored desiccated until use.

2. Horse Serum Butyrylcholinesterase (E-BChE)

E-BChE was purchased from Sigma Chemical Company (St. Louis, MO) Catalog Nos. C7512 and C4290. The enzyme activity in No. C7512 was reported to be 8.7 Units/mg powder. This powder was used at BIOTEK as the reference for measuring BChE activity. Sigma No. C4290 was reported to contain 610 Units/mg powder.

3. Bovine Serum Albumin (BSA) and Rabbit Serum Albumin (RSA)

Bovine Serum Albumin was purchased from Sigma Chemical Company, Catalog No. A-6793) Lot No. 10H0267 Rabbit serum albumin was also purchased from Sigma Chemical Company, (Catalog No. A-9438) Lot # 69329.

4. Suppocire-D

Suppocire-D, a semi-synthetic glyceride, was purchased from Gattefosse (Westwood, NJ) Lot No. 3502.

5. Triglyceride

Dynasan, a microcrystalline triglyceride was procured from Dynamit Nobel Chemicals (Rockleigh, NJ).

6. Poly Lactide-co-glycolide Polymers

Four polymers procured from Birmingham Polymers, Inc. (Birmingham, AL) were used for this project: 75/25 poly (D/L-lactide-co-glycolide), Lot No. 101-100-1, 65/35 poly (lactide-co-glycolide), Lot No. 115-62-1, 85/15 poly (Lactide-co-glycolide), Lot No. 112-31-2, and 50/50 poly lactide-co-glycolide, Lot No. 107-21-2.

B. Methods of Microsphere and Microcapsule Preparation

1. Preparation of Microspheres by Water-in-Oil (W/O)

A method of preparing microspheres containing water soluble drugs and proteins was developed at BIOTEK. This method consisted of dissolving the protein (and, in one case, lactose) in distilled water and adding the solution to calcium sulfate to form a "slurry". This "slurry" was poured into a beaker containing vegetable oil and fitted with a propeller blade for stirring. The propeller speed was varied. After one hour the solution was poured through sieves in a range from 300 μm and 25 μm to collect all microspheres in that range. Upon collection the residual oil was removed by washing with hexane or ethyl acetate. The processing was performed at room temperature.

2. Preparation of Microcapsules by Solvent Evaporation Water-in-Oil-in-Water (W/O/W)

Butyrylcholinesterase was dissolved in distilled water and the solution was emulsified into an organic polymer solution using a homogenizer. This water-in-oil emulsion was then emulsified into an aqueous media containing a surfactant. The microspheres were then collected, dried, sieved into fractions in the range 300-25 μm, and dried.

3. Sealing of Microspheres

Microspheres prepared by the water-in-oil (W/O) method can be sealed with a triglyceride to slow the release of protein and the dissolution of the microsphere and to help prevent breakage in the polymer overcoating process described below. An application of glyceride of about 10% (W/W) has been found effective.

The sealing is accomplished by placing microspheres into a maurumerizer. The sealant (Suppocire-D or Dynasan) is dissolved in ethylacetate. As the maurumerizer turns, the coating solution is added in aliquots. When the solvent from one aliquot has evaporated another aliquot is added, until the total volume has been applied.

4. Polymer Overcoating of Mineral Microspheres

Microfluidized bed microencapsulation is a process developed by BIOTEK for encapsulating small quantities of rare and labile drugs, pharmaceuticals, and other chemicals. A unique microfluidizer developed and built at BIOTEK requires a loading of only 5 grams, and even smaller quantities of drug have been coated using specialized processing conditions.

Microspheres in a selected range (105-150 μm for example) are placed in the microfluidized bed unit and converted into microcapsules by atomizing a solution of polymer in acetone and coating the microspheres to form a rate controlling capsule wall. The microcapsules are collected and sieved to collect the size range. The process is illustrated in **Figure 1**.

C. Analytical Methods

1. Measurement of BChE Activity

BChE activity was measured following the microwell assay method of **Doctor, et al. (1987)**. Standards of BChE were prepared. The substrate was a mixture of butyrylthiocholine (BTC) and dithiodinitrobenzene (DTNB). A colorimetric reaction occurs when the enzyme hydrolyzes BTC and the thiol released reacts with DTNB, producing the colored thionitrobenzene compound. Some reaction occurs even without enzyme, so the substrate mixture is used as the blank in the assay. The correlation of concentration vs. absorbance at 405 nm was linear over the range 0.0947-0.947 U/ml at an incubation time of 10 minutes (**Figure 2**). The exact procedure was as follows:

1) A stock solution of BChE was prepared with 50 mM pH 8.0 sodium phosphate buffer with 0.02% sodium azide.

2) Standards were made from the stock solution in the range from 0.0922 U/ml to 1.11 U/ml.

3) The Ellman assay reaction mixture consisted of 0.5 mM BTC-1 mM DTNB, 0.01% gelatinhydrolysate, and 0.2 M Tris (pH 7.6). The mixture was made up without BTC and stored in an amber-colored glass bottle at 4°C. Just prior to use, the appropriate amount of BTC was added from a 50 mM stock solution in distilled water.

4) Ten μl aliquots of samples or standards were added to wells of a 96-well microtiter plates (Corning ELISA Wells #24106-8).

5) To each of these wells, 200 μl of the Ellman reaction mixture was added using an 8-port micropipetter.

6) The microtiter plate was placed in the reader and read six times two minutes apart at a wavelength of 405 λ. The rate of change in absorbance for each well was calculated from this data.

7) The standards data were then plotted on a linear/linear scale, and the equation for the curve is used to calculate the concentrations of the unknowns.

2. Protein Content of Solutions by Micro Lowry Assay

The protein-containing microspheres were characterized by protein content (Micro Lowry Test). The Micro Lowry Protein Assay was carried out as follows: The standard used was a BSA solution (Sigma Catalog No. 690-10). The standard curve is made at concentrations from 5-25 mg/100 ml. The other reagents were: Biuret Reagent - Sigma Catalog No. 690-1 (0.75 mmol/L cupric sulfate and 0.4 mmol/L sodium hydroxide, with tartarate, iodide, and carbonate); Folin and Ciocalteu's Phenol Reagent - Sigma Catalog No. 690-2; Sodium Chloride (0.85%) for diluting standards and samples.

The procedure was as follows. A Corning Microwell Plate (24106-8) was used. At least two wells per plate were left blank. For each standard or sample, 0.040 ml of standard or sample was pipetted into a micro centrifuge tube. Then 0.440 ml of Biuret Reagent was added to the tube and the contents were mixed well. After ten minutes, 0.020 ml of Folin and Ciocalteu's Phenol Reagent was added with mixing. After thirty minutes, 0.200 ml of solution was pipetted into at least two wells per sample. The plate was read at 630 nm on a Bio-Tek Microwell Plate Reader and the concentrations of the samples were calculated from the standard curve. A graph of a standard curve for the concentration range 0.05-1.00 mg/ml is shown in Figure 3.

3. Protein Content by UV Absorbance

An ultraviolet scan of butyrylcholinesterase in distilled water showed a UV maximum of 276 nm. Based on a standard curve, concentrations of E-BChE in water or buffer were calculated based on absorbance at 276 nm.

4. Extraction of Protein from Microspheres and Microcapsules

a. Extraction from Polymeric Microspheres

Assay for enzyme content of polymeric microcapsules and microspheres was performed by dissolving an accurately weighed quantity of the microparticles in methylene chloride and extracting the enzyme from the organic phase with distilled water and measuring the absorbance at a wavelength of 276 nm. Polymer absorbance in the UV is minimal.

b. Extraction from Calcium Sulfate Microspheres

To release protein from calcium sulfate microspheres, the medium of choice was distilled water. The reason for this is that buffer reagents such as phosphate can react with the components of the microspheres. Calcium sulfate is relatively insoluble in water (0.3 mg/ml) so a volume sufficient to dissolve the spheres would produce a very dilute solution of microspheres from low loading spheres. Thus, a method of protein removal which did not involve complete dissolution of the particles was developed. The general procedure for recovery of protein from calcium sulfate microspheres was to place 10 mg of microspheres in 20 ml distilled water for at least 24 hours at room temperature.

c. Removal of Sealant and Polymer Overcoat from Calcium Sulfate Microspheres

It was expected that the above method would not be suitable for removing protein from sealed and coated calcium sulfate microspheres. After all, the purpose of the coating is to retain the protein. Thus, a method of removing the coating and sealant while not affecting the protein was established. Aliquots of sealed microspheres or coated microcapsules were placed in tared jars. The jars were then reweighed. The samples were rinsed three times with methylene chloride, which was added and removed from the container with a disposable pipette. The samples were air dried, then the containers were reweighed to obtain the amount of organic material removed. The samples are then treated as in the previous section to release the protein from the bound matrix.

5. Size Distribution

Microcapsules size distribution for each run was determined by sieve analysis through a series of sieves with decreasing mesh size, using an Allen-Bradley sonic sifter to insure complete separation of the microcapsules.

6. In Vitro Release

In vitro cholinesterase release was determined by placing 20 mg sample of microparticles in a heat sealable pouch made from polypropylene fabric with 30 μm pore size. The fabric allows free access of fluids without loss of microparticles during elution medium replacement. The microparticle-loaded pouch was suspended in water or 0.04 M pH 7.4 phosphate buffered saline (PBS) at 37°C. The mixture was placed in a shaker bath (shaking at 98 rpm) in a hot box at a constant temperature of 98°F. At given intervals, an aliquot of either water or buffer was removed or the entire solution was exchanged for determination of cholinesterase concentration. Measurement of release rate was performed regularly until the total amount of drug released approached 100% of the assayed drug content of the microcapsules. The enzyme concentration in the elution medium was determined either by UV absorption at 276 nm or by enzyme assay, or by micro Lowry assay.

7. Measurement of Calcium Ion Release

Dissolution of microspheres and microcapsules was measured by release of calcium ion. Aliquots of the materials tested were placed in mesh bags and suspended in 65 ml distilled water at 37°C. Periodically, the water was collected for analysis and the bags were placed in fresh distilled water. The concentration of calcium ion was measured with a Corning 476041 calcium ion specific electrode.

IV. RESULTS AND DISCUSSION

A. Butyrylcholinesterase Clearance

1. Intravenous Clearance

Two rabbits were injected I.V. with 2000 U E-BChE. The analysis of the level of BChE in plasma was conducted at WRAIR. The results, which were supplied by Ms. Mary Kay Gentry at WRAIR are shown in **Table 8A**. The blood data was analyzed using PCNONLIN Version 3.0, a software for the statistical analysis of pharmacokinetic models.

Most drugs follow two compartment pharmacokinetics (i.e.) a distribution phase from the blood into the tissues followed by an elimination phase **(Figure 4)** for model description. The 168 hour data point was eliminated from the analysis because it was an outlier. The plasma profile in a two compartment model is described by the nonlinear relationship:

$$C_{(t)} = A\,e^{-\alpha t} + B\,e^{-\beta t}$$

where,

$C_{(t)}$	=	is the plasma concentration of BChE at time (t).
A	=	Intercept of the distribution phase.
B	=	Intercept of the elimination phase.
α	=	First order rate constant of the distribution phase.
β	=	First order rate constant of the elimination phase.

Using this model, the following values were computed for the above equation.

$$C_{(t)} = 231.99\,e^{-4.20t} + 13.70\,e^{-0.011442t}$$

A plot of this equation is depicted by the solid line in **Figure 5**. The data points in the plot represent the actual plasma level. The correlation between the two sets of data is very good (r^2 = 0.985). There are only two points which describe the distribution phase. Despite this, the program computed the pharmacokinetic parameters listed in **Table 9**.

The plot in **Figure 5** and the data in **Table 9** show that E-BChE has a tendency to remain in the tissue. Note, for example, that the half life of the elimination phase (Beta-HL) is more than sixty hours. This may be due to binding of the enzyme to tissue. Also, the ratio of K21/K12 is 0.24/3.77 or 0.06 which means that the enzyme is being eliminated from the tissues at 6% of the rate it is coming into the tissues.

2. Intramuscular Clearance

Two rabbits were injected I.M. with 2,200 U E-BChE. The analysis of the level of BChE in plasma was conducted at WRAIR. The results, which were supplied by Ms. Mary K. Gentry at WRAIR, are shown in **Table 8B**. The blood data was analyzed using PCNONLIN Version 3.0, a software for the statistical analysis of pharmacokinetic models. The intramuscular clearance data confirms the intravenous findings. **Figure 6** is the model used to analyze the data. This model assumes first order input (instead of the bolus input used in the I.V. model) and a first order output. The final values are shown in **Figure 7** and follow each of the plasma data points. The final equation looks like this:

$$C_{(t)} = A\,e^{-\alpha t} + B\,e^{-\beta t} + C\,e^{-(K01)t}$$

where,

C	=	- (A + B)
K01	=	Absorption rate

and the actual values are:

$$C_{(t)} = 17.65\,e^{-0.034891t} + 27.12\,e^{-0.014456t} - 44.77\,e^{-0.027177t}$$

Again here, the correlation between the data and the calculated plot is very good (r^2 = 0.997). The plot shows very slow distribution of the enzyme from the muscle and the plasma level takes 58.42 hours to reach the maximum level of 4.8 U/ml **(Table 10)**. The elimination half life (K10-HL) is 66 hours which is very close to the 60 hours computed in the I.V. study.

B. Repeated Long-Term Butyrylcholinesterase Injections and Cross-Over to Acetylcholinesterase Injections

1. Results

Five rabbits were each given a total of ten injections of E-BChE over a period of twenty months. Blood was collected at frequent intervals immediately after injections and every two weeks until the next injection. The five rabbits were maintained on the routine for up to 617 days. On day 617, the cross-over study started with I.M. injections of FBS-AChE on days 617, 620, and 624. On day 624, the five rabbits plus one naive rabbit were given an I.V. injection of FBS-AChE. The following day 3 rabbits were found dead and one rabbit moribund. The two remaining rabbits were test bled on days 696, 698, and 701 and then exsanguinated on day 703. The blood samples were analyzed for cholinesterase activity and anti-E-BChE levels by Ms. Mary K. Gentry at WRAIR. Weights of the rabbits are given in **Table 11**.

The mean blood BChE levels of five rabbits and anti-E-BChE IgG levels measured throughout the four series of injections of E-BChE are shown in **Figures 8A through 8D**. Series One consisted of four injections, given on days 1, 4, 8, and 11. The blood BChE level was raised from a baseline of about 0.2 Units per ml to a maximum of 2.1 Units per ml on day 6. When the second series of E-BChE injections was given after about a three month interval, on days 127 and 130, the elevation of blood BChE activity was much less than the elevation achieved with the first injection. The highest level measured was only 0.5 Units/ml. After an interval of almost 5 months the third series of E-BChE was given on days 274 and 275. A peak blood BChE level of 1.2 Units per ml was found. The last injections of BChE were given after an interval of six months on days 456 and 457. No significant elevation of blood BChE activity was found.

After the first series of injections, blood anti-E-BChE IgG developed at a low level, peaking at 1.0 mg/ml on day 5. After each subsequent series of injections the levels rose higher and were elevated for a longer duration. The peak anti-E-BChE after the second injection series was 13 mg/ml; after the third series it was 19 mg/ml and after the fourth series it was 24 mg/ml.

When the injections of FBS-AChE were given to these rabbits five and a half months after the last E-BChE injection, no elevation of AChE activity over baseline was observed. A small increase in blood anti-FBS-AChE IgG was seen, as well as a moderate increase in blood anti-E-BChE IgG (6.9 mg/ml). The mean blood levels of BChE, AChE, anti-E-BChE IgG, and anti-FBS-AChE IgG are shown in **Figure 8E**.

After the cross-over injections of I.V. FBS-AChE three rabbits died within 18 hours. Three were euthanized, two in extremis. Gross necropsies were performed on all six rabbits and the following tissues were collected for histopathology, at the Contract Officers Technical Representative's request: heart, liver, spleen, kidney, urinary bladder, retropharyngeal lymph node, and mesenteric lymph node tissue.

Gross necropsies for two rabbits which had been euthanized (Nos. 99 and 443) indicated no abnormalities. One of the three rabbits which had been euthanized was found to have diffuse congestion of subcutaneous vasculature with suggestion of hemorrhage of numerous vessels, but no other abnormalities.

The three rabbits which were found dead had blood in the fur at the external nares, congested subcutaneous vasculature, post mortem discoloration of GI tract and uterine horns or uterus, and two had hearts distended with blood. Two rabbits had red retropharyngeal lymph nodes and in one of these the mesenteric lymph nodes were also red. Yellow mottling or foci on the livers and red mottling on the lungs was observed in two of these rabbits. Soft kidneys were reported in two rabbits. These necropsies were performed at Massachusetts Institute of Technology Department of Comparative Medicine by a veterinary pathologist. The reports are included in Appendix A.

It is unfortunate that lung tissue was not also taken for histopathology, as in none of the above samples was any probable cause of death or morbidity observed. The most significant histopathology findings were lymphoid depletion in four rabbits and lymphoid follicular hypertrophy/hyperplasia in 2 rabbits. Increased hemosiderin pigment within splenic macrophages was seen in four of the rabbits. The reports by the histopathologist are included in Appendix A.

2. Discussion

Using equine enzyme in rabbits in the repeat injection study resulted in the development of antibody responsiveness which could neutralize subsequently injected enzyme, even after several months. This conclusion is based on the decreasing activity found after each series of injections and the increasing antibody response to each injection series. Throughout the study a baseline level of blood BChE was maintained, illustrating that the antibody response had not been directed at rabbit cholinesterases. A slight cross-reactivity of the anti-E-BChE IgG to FBS-AChE was observed.

C. Repeated Long-Term Acetylcholinesterase Injections and Cross-Over to Butyrylcholinesterase Injections

1. Results

Five rabbits were each given a total of seven I.M. injections of FBS-AChE over a period of sixteen months. Blood was collected at frequent intervals immediately after injections and every two weeks until the next injection. The study continued up to day 583. At day 597, the cross-over study started with a single I.M. injection of E-BChE. Blood samples were drawn on days 598, 599, 626, and rabbits were euthanized on day 631. Butyrylcholinesterase (3,500 U) was given I.M. on June 13, 1995. The blood samples were analyzed for cholinesterase activity and anti-FBS-AChE levels by Ms. Mary K. Gentry at WRAIR. Weights of the animals, taken periodically, are shown in Table 11.

Figures 9A and 9B illustrate the mean levels of AChE and anti-FBS-AChE antibodies found in the blood of five rabbits. The first injection produced significantly increased AChE in the blood. A peak level of approximately 13 U/ml was achieved on day 8. The level quickly dropped to near zero by day 15. Subsequent injections caused very small increases in the AChE levels, while the AChE antibody level increased sharply and maintained an increased level for over 200 days.

The five rabbits were euthanized at the end of the study. Gross necropsies were performed on all five rabbits and the following tissues were collected for histopathology, at the Contract Officers Technical Representative's request: heart, liver, spleen, kidney, urinary bladder, retropharyngeal lymph node and mesenteric lymph node tissue. Aside from a mass on the left uterine horn of one rabbit, the gross necropsies showed no abnormalities. These necropsies were performed at Massachusets Institite of Technology Department of Comparative Medicine by veterinary pathologists. The reports are included in Appendix A.

A histopathological examination of the tissues collected showed no evidence of a disease process or toxicologic activity. The most significant findings were varying degrees of lymphoid depletion and follicular hypoplasia in the spleens of all five rabbits and varying levels of increased hemosiderin pigment within splenic macrophages in four of the rabbits. There was slight hepatocyte vacuolation in two rabbits and increased glycogen deposition in hepatocytes in one rabbit. All other tissues examined appeared normal. The reports by the histopathologist are included in Appendix A.

2. Discussion

The results of this study were similar to those obtained with the repeated injections of E-BChE. The first injections of FBS-AChE produced high levels of blood AChE activity. A rise in anti-FBS-AChE antibodies peaked at day 28 and dropped to baseline by day 42. After the second and third series of injections, only small increases in blood enzyme level were observed, and the antibody response was stronger and more persistent, not dropping to baseline for several months. When the cross-over injection of BChE was given, a high level of blood BChE was seen, showing the lack of cross-reactivity of the antibody. No antibody elevation (to either E-BChE or FBS-AChE) was found after the BChE injection.

D. Production of Polyclonal Antibodies Against Natural and Synthetic Peptides

1. Study One Results

In this study rabbits were injected with peptide liposomes. There were fourteen peptides and each peptide was used to immunize two rabbits. Weights of the animals, taken periodically, are shown in <u>**Table 12**</u>. Test bleeds from the rabbits were analyzed for the presence of antibody to the peptide which the rabbit had received. The liposomal preparations did not generate a significant level of polyclonal antibodies to the peptides.

2. Study Two Results

In this study rabbits were injected with peptides conjugated to proteins. There were thirteen peptides and three rabbits were immunized with each peptide. Weights of the animals, taken periodically, are shown in <u>**Table 13**</u>. Test bleeds from the rabbits were analyzed for the presence of antibody to the peptide which the rabbit had received. The results, provided by Ms. Mary K. Gentry at WRAIR, are reproduced in <u>**Figures 10A through 10F**</u>.

3. Discussion of Study Two

In every case but one, either no antibodies or a very low level of antibodies against the inoculum were found in the control sera. The exception was Rabbit Number 102, in the group receiving peptide FLIP. This rabbit, which had participated in the E-BChE clearance study, had positive response at up to 3.5 log dilutions before <u>first</u> dose and a more elevated response after the injection. The other two animals in the FLIP group differed in that one had no significant response and the other had a positive response up to 3 log dilutions after the first injection.

Peptides FBS1, FBS2A, FBS3, CHE5, and CD43 were similar in that all three animals in each group had very low response after the first injection, but the sera collected after the booster were positive at up to 3.5 log dilutions. In peptide CHE3, the response, even after the booster, was weak. Peptides CHE2 and CD41 gave no response in the first sera samples but two of the three were positive at up to 3.5 log dilutions after the booster. Peptides FBS4 and CHE1 each produced a weak response after the booster in one animal. Peptide CD42 induced a positive response up to 3.5 log dilutions in one animal after booster and peptide CHE4 did not induce a significant positive response.

E. Induction of Anti-Idiotypic Antibodies to Fab Fragments from Anti-FBS-AChE Monoclonal Antibodies

1. Results

Three rabbits were given primary immunizations and two booster immunizations with 25B1, a Fab fragment from a monoclonal antibody to AChE and three rabbits were given the same series of immunizations with 13D8, another Fab fragment from a monoclonal antibody to AChE. Test bleeds were taken before injection and after each injection. The bloods were analyzed at WRAIR for antibody to the Fab fragments. Figures 11A through 11C show the titers against 25B1 after the initial, second, and final injections. Figures 11D and 11E show the titers against 13D8 after the initial and final injections. Weights of the animals, taken periodically, are shown in **Table 14**.

2. Discussion

Each rabbit developed antibodies to the Fab fragment. It was interesting, however, that the titers did not increase after the booster injections.

F. Microsphere and Microcapsule Formulations

1. Results

a. Water-in-Oil (W/O) Formulations

1) Preparation of Calcium Sulfate Microspheres

Thirty formulations were prepared using BIOTEK's water-in-oil (W/O) method. Table 15 lists the W/O formulations, their components, and the results of tests performed on each batch. These tests include Micro Lowry Protein Assay, E-BChE activity (assay for enzyme), in vitro release, scanning electron microscopy (SEM), and calcium ion release.

Early formulations were prepared with a low potency E-BChE, which was successfully added at a loading of 9%. An example is 88MS15. An SEM of these microspheres is shown in Figure 12A. When the methodology was used to prepare formulations from a much higher potency enzyme, a significantly smaller amount of enzyme powder was used (0.4%). Under these circumstances no enzyme activity was recovered from the formulations. Two approaches to solving this problem were evaluated.

In Formulation 72MS16, lactose was added as a filler and stabilizer, however this was not beneficial. A lower level of activity was found. Because BChE is known to adhere to surfaces readily, bovine serum albumin (BSA) was added to formulations to protect the E-BChE from denaturation and from adhering to the microsphere surface. This approach did increase the recovery of BChE activity in the high potency formulations from 0% to more than 20% in 72MS15.

In addition, non-ionic surfactants (i.e. Mannitol, Tween 20, Tween 80, and glycerol) were evaluated as aids in strengthening the microspheres. Microspheres prepared with glycerol were well formed and stronger than those made without glycerol. It seemed that these microspheres would be strong enough to be overcoated with polymer without application of the sealant. In order to obtain sufficient glycerol strengthened microspheres to test this hypothesis, ten formulations containing BSA [72MSG6-10] were prepared under scale-up conditions. Several preparations were needed as the yield in the desired size range was low. The microspheres from the five batches were pooled (17MSB1) and overcoated. The overcoating is described in the next section.

Subsequently, mannitol was shown to be useful in increasing the yield of microspheres obtained in the size range 105-150 μm. Batch MSG15 illustrates this improved yield. The mannitol was 10% of the liquid component.

The glycerol and BSA stabilized water-in-oil microspheres were the model for the material prepared for in vivo testing. In order to prepare high potency E-BChE microspheres for in vivo testing in rabbits, rabbit serum albumin was used in place of bovine serum albumin (to avoid immune reaction in the rabbit). Three batches of microspheres were prepared (using 1-25% glycerol/water as the liquid component) in order to obtain sufficient microspheres in the desired size range for the entire in vivo series planned. Batches [72MSG2, 3, 4, & 5] were each prepared with about 10% total protein, including both rabbit serum albumin and E-BChE. The target activity was 6 U BChE/mg microspheres. The resultant microspheres were round and smooth. An SEM of 72MSG5 is shown in **Figure 13**. The microspheres in the size range (75-150 μm) from these batches were pooled, yielding a total of 2.025 g. These microspheres, which had an activity of 3 U BChE/mg, were used in a repeat injection study in rabbits.

b. Sealing and Overcoating of Water-in-Oil (W/O) Calcium Sulfate Microspheres

Since the water-in-oil calcium sulfate microspheres have a high surface area and release protein quickly, it was desirable to coat them with a partially permeable polymer membrane. **Table 16** lists the seven formulations which were made by sealing and/or over-coating the microspheres. The table gives information on components, and the results of tests performed on each batch. These tests include Micro Lowry Protein Assay, E-BChE activity (assay for enzyme), in vitro release, surface morphology, and calcium ion release.

It had been previously found that calcium sulfate microspheres break during fluidized bed coating. Around 50% of the starting material is reduced to a fine dust which deposits on the filters. This problem was initially solved by applying a triglyceride sealant to the microsphere cores (72W1-3). The pores in the protein loaded calcium sulfate microspheres were filled with triglyceride, producing a smoother and stronger material. **Figure 12B** illustrates the change from porous to filled core in the sealed 88MS15. **Figure 12C** illustrates the polymer overcoated core (88W1) and **Figure 12B** illustrates the durability of the overcoating shell as the SEM is taken after the microspheres had been in water for 38 days. **Figure 14** shows the release of protein and calcium from 88W1. Notice how closely the calcium ion release duplicates the protein release.

Another solution to the problem of breakage during overcoating of the microsphere cores was to make the microspheres less fragile and less susceptible to drying. It was expected that the glycerol containing microspheres could be over-coated directly without sealing. This hypothesis was tested by overcoating 7 g of 10% BSA-loaded microspheres (72MSB1) prepared with 2.5% glycerol (pooled from preparations [72MSG6-10] in the size range 75-150 μm). The overcoating was with a 65:35 PLLA-PGA and the target coating level was 10%. The glycerol content did reduce the microsphere fragility, however there was still some breakage, leading to contamination of the coated microspheres with fragments [72W4]. The yield on the process was 67.5 % of theoretical weight.

The uncoated microspheres (72MSB1) released calcium very quickly, almost 100% in 1-2 days. But after overcoating with PLGA polymer, the release slowed down dramatically. **Figure 15** shows that 95% of the calcium was released in 149 days in Batch 72W4. As we have shown that protein release closely resembles calcium ion release, it is clear that such a system could provide very long term enzyme delivery.

c. Water-in-Oil-in-Water (W/O/W) Formulations

Another approach to preparing microspheres with calcium sulfate cores and polymer shells is the W/O/W (water/oil/water) method. Eighteen formulations were prepared using this method. **Table 17** lists the W/O/W formulations, their components, and the results of tests performed on each batch. These tests include Micro Lowry Protein Assay, E-BChE activity (assay for enzyme), in vitro release, surface morphology, and calcium ion release.

SEM's reveal microspheres that are smooth, spherical, and well-coated with polymer. For example a SEM of 72MS18 is shown in **Figure 16**. Formulation 72MS18 was prepared with 90% retention of activity. Release data of both protein and calcium from 72MS18 **(Figure 17)** indicates the release from this formulation was too rapid. Slower release may be achieved with the use of a larger proportion of polymer. Microcapsules 72MS27 released calcium for 21 days and microcapsules prepared with the addition of calcium phosphate (72MS26) released calcium for 49 days **(Table 17)**.

2. Discussion

BIOTEK prepared E-BChE delivery systems by two novel methods which utilized porous inorganic cores for protein incorporation. In the two-phase method (W/O), microsphere cores were preformed without the use of organic solvent, then microcapsules were formed by overcoating the microspheres with polymer. In the simpler three-phase method (W/O/W), the protein was incorporated in the mineral matrix at the same time that the polymeric shell was formed. Active enzyme was recovered from products prepared by both methods. It was shown that the duration of protein release and dissolution of the mineral core could be controlled by the amount of applied polymer coating. The most important advantage of the new process is that high protein loadings can be achieved. A second advantage is the reduced exposure to organic solvents. Uncoated E-BChE microspheres prepared by the W/O method were tested in vivo, as described in the next section. Blood levels of BChE were elevated, as were antibodies against E-BChE. The uncoated microspheres seemed to exhibit adjuvant properties. Polymer coated microcapsules would be more suitable for an enzyme delivery product.

G. Repeated Injections of E-BChE or E-BChE Microsphere Solutions

1. Results

Three rabbits were injected with microspheres containing E-BCHE. Each dose contained , 667 U E-BChE (referenced to the activity reported by Sigma). The injections were repeated twice at weekly intervals, with the same dose sizes being given. As controls, three rabbits were give injections of E-BChE solution, 667 U/injection on the same schedule as the injections of microspheres. Test bleeds were taken before the first injection and regularly thereafter for 43 days. The blood samples were sent to Ms. Mary K. Gentry at WRAIR for determination of cholinesterase activity and anti-E-BChE antibody levels. A sample of the enzyme solution used was also sent to Ms. Gentry so that a correlation of Sigma Chemical Company activity Units with those measured by WRAIR could be made. According to the WRAIR assay, the potency of the E-BChE was 22% of the value given by Sigma. Thus based on the WRAIR assay, the doses given were each 146.7 U each. The mean blood BChE levels for each group are shown in **Figure 18A** and the mean anti-E-BChE IgG levels are shown in **Figure 8B**. Rabbit weights, taken periodically, are shown in **Table 18**.

2. Discussion

The highest blood enzyme levels achieved with the **microsphere injections** were lower than those in the animals which were dosed with enzyme solution. Since the microspheres were designed to control the release of the enzyme, it was expected that a lower maximum enzyme level but a prolonged duration of elevated enzyme level would be obtained. Also, a portion of this decrease was probably due to the hold-up of some microspheres in the syringe. The mean blood levels of the group receiving the microsphere injections was significantly higher (t-Test Paired Two-Samples for Means, Two-tail $P >= 0.05$), only at 72 hours after the first injection. The mean BChE levels dropped in the second week. This correlated with the rise in blood anti-E-BChE IgG beginning around day 8.

During the week after the first injection, the blood BChE levels increased significantly (t-Test Paired Two-Samples for Means, Two-tail $P >= 0.05$) at 1, 6, 24, 48, 72, and 96 hours in the group receiving **solution injections**. By 168 hours the levels were not different from the pre-injection level. There was a slight but not significant increase after the second injection of enzyme solution (at 196 hours) but thereafter, even after the third injection, there was no significant change from pre-injection levels. This data also correlated with the increase in antibody which may have neutralized these injections.

Unfortunately, there was a large variability in pre-injection blood BChE levels (in a total of 15 rabbits in 4 studies the mean was 0.209 ± 0.127 U/ml and in this recent study with six rabbits the mean was 0.225 ± 0.142 U/ml). With this much variability, small changes were not significant.

The results with solution injections were compared with data obtained from a similar study in Phase I. The mean blood cholinesterase levels obtained in the two studies are shown in **Figure 19**. The mean blood BChE levels achieved as a result of the three solution injections of 147 U (based on the WRAIR assay) reached a maximum of about 0.75 U/ml, whereas in the earlier work with WRAIR-purified material during a series of five injections of 420 U each, the highest mean blood BChE level found was 1.5 U/ml. The more frequent dosing appears to have maintained the blood enzyme levels longer than the once a week regimen, as would be expected. After one week, however, additional doses were not effective.

V. CONCLUSIONS

A. Success in Meeting Objectives

BIOTEK was successful in meeting the four program objectives.

To meet the first objective of "following the long term effect on the immune system of repeated injections of cholinesterases", studies were carried out in rabbits with two enzymes (E-BChE and FBS-AChE) for nearly two years. During this period, the rabbits were healthy and test bleeds and injections were carried out as specified by the project officer, Ms. Mary Kay Gentry at WRAIR. Blood specimens were prepared and shipped regularly to Ms. Gentry, for enzyme and IgG determination. This data is included in the report. In addition, BIOTEK carried out pharmacokinetic clearance studies of E-BChE after single I.M. or I.V. injections in rabbits. The blood specimens from these studies were also analyzed by Ms. Gentry and the data was used at BIOTEK to calculate the pharmacokinetic parameters of E-BChE.

The second objective, "to produce rabbit antibodies against natural and synthetic peptides" was also met. Two series of antisera production were carried out. In the first series the peptides were provided by WRAIR to BIOTEK in liposomal formulations. BIOTEK carried out the injections in rabbits and collected blood samples and antisera. The blood was analyzed at WRAIR for the presence of antibodies to the peptides. The liposomal formulations did not produce useful levels of antibodies. In the second peptide injection series, the peptides were conjugated to protein and administered in rabbits with a potent adjuvant (Freund's Complete Adjuvant). This series was successful and antisera provided by BIOTEK to WRAIR contained antibodies to the peptides.

The third objective "preparation of antisera against fragments of the Fab portion of monoclonal antibodies against FBS-AChE" was met by BIOTEK. The Fab fragments provided by WRAIR were injected into rabbits, which were maintained in a healthy condition until antisera were collected and shipped to WRAIR.

The final objective, "the evaluation of injectable slow-release butyrylcholinesterase formulations" was also met. Many formulations of E-BChE were prepared and evaluated in vitro for duration of release and retention of enzyme activity. Based on those results, one formulation was evaluated in vivo in rabbits for 43 days. During this period, blood specimens were collected and shipped to Ms. Mary K. Gentry at WRAIR for measurement of blood enzyme levels and also levels of blood antibodies to E-BChE. The results presented in this report show elevated levels of E-BChE in blood, consistent with the slow release of E-BChE from the formulation. Antibody to E-BChE was also found in the rabbits, consistent with the release of intact enzyme from each microsphere injection over a period of one or two weeks.

B. Implications for Future Work

The work with injections of equine butyrylcholinesterase and fetal bovine acetylcholinesterase in rabbits has shown that substantial elevation of blood cholinesterase levels can be obtained with a single enzyme injection. Furthermore, the enzyme persists in the blood for several days after the first injection. This result is very promising because of its implications for administering the enzyme as a prophylactic to individuals who could be exposed to deadly organophosphate agents.

It is also apparent that when enzymes from other species are injected in rabbits, antibodies specific to the heterogeneous enzymes are generated. Such antibodies appear at low levels within seven days of the first injection. Even after a period of several months, subsequent injections stimulate rapid elevation of antibody to the heterologous enzyme and they apparently neutralize the enzyme quickly as the levels attained from injections given after 4-5 months are not as high as those seen after the initial injections.

Assuming that a similar response would be seen in humans, this data indicates that heterologous enzymes can be successfully employed on a one time basis for protection. If the mode of treatment is repeated, however, the formation of antibodies to the enzyme would be deleterious. With the use of heterologous enzyme, there could be little confidence in the efficacy of the treatment.

This problem may well be solved with the use homologous enzyme for prophylactic treatments. A continuing program, beginning with the use of rabbit cholinesterases in rabbit studies, followed by studies of the use of homologous enzyme in primates would be the logical prelude to a clinical study with human enzyme.

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TABLE 1

LONG-TERM REPEATED BChE AND AChE CROSS-OVER SCHEDULE

FOR RABBIT NOS. 96-100 (and 443 from Day 694 Onward)

BChE INJECTIONS				BLOOD DRAWING (Test Bleeds)	
STUDY DAY	ROUTE	DOSE	DATE	STUDY DAY	DATE
1	I.M.	1,273U BChE	21-Jun-93	1 Pre-injection	21-Jun-93
4	I.M.	1,273U BChE	24-Jun-93	2	22-Jun-93
8	I.M.	1,273U BChE	28-Jun-93	5	25-Jun-93
11	I.M.	1,273U BChE	01-Jun-93	9	29-Jun-93
				12 and every 2 weeks	02-Jul-93
127	I.M.	1,527U BChE	25-Oct-93	until	
				128	26-Oct-93
130	I.M.	1,527U BChE	28-Oct-93		
				131	29-Oct-93
				138 and every 2 weeks	05-Nov-93
				until	
274	I.M.	1,500U BChE	21-Mar-94	275	22-Mar-94
275	I.M.	1,500U BChE	22-Mar-94	276	23-Mar-94
				277	24-Mar-94
				281	28-Mar-94
				289 and every 2 weeks	05-Apr-94
				until	
456	I.M.	1,409U BChE	19-Sep-94	457	20-Sep-94
457	I.M.	1,409U BChE	20-Sep-94	458	21-Sep-94
				459	23-Sep-94
				463	26-Sep-94
				470 and every 2 weeks	03-Oct-94
				until	
617	I.M.	1,750U AChE	27-Feb-95	618	28-Feb-95
620	I.M.	1,750U AChE	02-Mar-95	621	03-Mar-95
624	I.M.	1,750U AChE	06-Mar-95	625	07-Mar-95
				631 and every 2 weeks	13-Mar-95
				until April 24, 1995	
694(1)	I.V.	5,000U AChE	15-May-95	694 (prebleed, 2 & 5 hrs)	15-May-95
				696(2)	17-May-95
				698	19-May-95
				701	22-May-95
				703 (exsanguinated)	24-May-95

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(1) An additional rabbit (No. 443) was added to the study to act as a naive rabbit.
(2) Nos. 96, 97, and 98 were found dead on day 695, and No. 100 was euthanized on day 696, due to being moribund.

TABLE 2

LONG-TERM REPEATED AChE SCHEDULE FOR RABBIT NOS. 105-109

INJECTIONS				BLOOD DRAWING	
STUDY DAY	ROUTE	DOSE	DATE	STUDY DAY	DATE
1	I.M.	370U AChE	25-Oct-93	Day 1 Pre-injection	25-Oct-93
				Day 2	26-Oct-93
4	I.M.	370U AChE	28-Oct-93		
8	I.M.	370U AChE	01-Nov-93	Day 5	29-Oct-93
11	I.M.	370U AChE	04-Nov-93	Day 9	02-Nov-93
				Day 12 and every 2 weeks until	05-Nov-93
148	I.M.	3,636U AChE	21-Mar-94	Day 149	22-Mar-94
149	I.M.	3,636U AChE	22-Mar-94	Day 150	23-Mar-94
				Day 151	24-Mar-94
				Day 155	28-Mar-94
				Day 163 and every 2 weeks until October 31, 1994	05-Apr-94
491	I.M.	1,527U AChE	27-Feb-95	Day 492	28-Feb-95
				Day 493	01-Mar-95
				Day 495	03-Mar-95
				Day 498	06-Mar-95
				Day 505 and every 2 weeks until April 24, 1995	13-Mar-95
				568	15-May-95
				583	30-May-95
597	I.M.	4,455U BChE	13-Jun-95	597 (prebleed, 2 & 5 hrs)	13-Jun-95
				598	14-Jun-95
				599	15-Jun-95
				626	12-Jul-95
				631 (euthanized; no test bleeds drawn)	17-Jul-95

TABLE 3

PEPTIDE IMMUNOGEN DESCRIPTIONS, GROUP CODES, AND RABBIT ASSIGNMENTS

PEPTIDE DESCRIPTION	GROUP CODE	RABBIT NUMBER	
		STUDY 1 (Protocol 134) (Peptides in Liposomes)	STUDY 2 (Protocol 176) (Peptide Conjugates)
Synthetic fragment of CD4 (31–59)	CD41	59, 60	231, 232, 104*
Synthetic fragment of CD4 (49–72)	CD42	61, 62	233, 234, 235
Synthetic fragment of CD4 (63–42)	CD43	63, 64	236, 237, 238
Synthetic fragment of Bovine AChE (223–247)	FLIP	65, 66	102*, 215, 216
NT Receptor Peptide 1	NTR1	67, 68	
NT Receptor Peptide 2	NTR2	69, 70	
NT Receptor Peptide 3	NTR3	71, 72	
NT Receptor Peptide 4	NTR4	73, 74	
NT Receptor Peptide 5	NTR5	75, 76	
Recombinant 55K fragment of CD4 cell marker protein	SCD4	77, 78	
Stonefish Toxin Cyanogen Bromide Cleared	XCNB	79, 80	
NT Receptor Peptide 8–13 Complex Aggregate	XN8	81, 82	
Purified Stonefish Venom Toxin	XNAT	83, 84	
Purified Stonefish Venom Toxin (inactivated)	XRDA	85, 86	
Cholinesterase Peptide 1	CHE1		217, 218, 103*
Cholinesterase Peptide 2	CHE2		219, 220, 221
Cholinesterase Peptide 3	CHE3		222, 223, 224
Cholinesterase Peptide 4	CHE4		225, 226, 227
Cholinesterase Peptide 5	CHE5		228, 229, 230
Fetal Bovine Serum AChE (55–98)	FBS1		101*, 204, 205
Fetal Bovine Serum AChE (264–299)	FBS2A		206, 207, 208
Fetal Bovine Serum AChE (321–362)	FBS3		209, 210, 211
Fetal Bovine Serum AChE (426–461)	FBS4		212, 213, 214
Control	KLH		239, 240, 241

* Used previously in the E–BChE clearance study

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TABLE 4

PEPTIDE STUDY ONE SCHEDULE

Animal No.	Code	Week 0 Prebleed Immunization	Week 4 Boost	Week 5 Test Bleed	Week 6 Exsanguinate	Week 8 Boost	Week 9 Test Bleed	Week 10 Euthanize	Week 10 Exsanguinate
59, 60	XNAT	01–Jun–93	29–Jun–93	06–Jul–93	--	27–Jul–93	02–Aug–93	10–Aug–93	--
61, 62	XRDA	01–Jun–93	29–Jun–93	06–Jul–93	--	27–Jul–93	02–Aug–93	10–Aug–93	--
63, 64	SCNB	01–Jun–93	29–Jun–93	06–Jul–93	--	27–Jul–93	02–Aug–93	10–Aug–93	--
65, 66	SCD4	01–Jun–93	29–Jun–93	06–Jul–93	13–Jul–93	--	--	--	--
67, 68	CD41	01–Jun–93	29–Jun–93	06–Jul–93	--	27–Jul–93	02–Aug–93	--	10–Aug–93
69, 70	CD42	01–Jun–93	29–Jun–93	06–Jul–93	--	27–Jul–93	02–Aug–93	#70 10–Aug–93	#69 10–Aug–93
71, 72	CD43	01–Jun–93	29–Jun–93	06–Jul–93	--	27–Jul–93	02–Aug–93	--	10–Aug–93
73, 74	FLIP	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	--	11–Aug–93
75, 76	XN8	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	--	11–Aug–93
77, 78	NTR1	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	--	11–Aug–93
79, 80	NTR2	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	--	11–Aug–93
81, 82	NTR3	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	--	11–Aug–93
83, 84	NTR4	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	--	11–Aug–93
85, 86	NTR5	02–Jun–93	30–Jun–93	07–Jul–93	--	28–Jul–93	03–Aug–93	11–Aug–93	--

196,2172–F\72–FT4

TABLE 5

PEPTIDE STUDY TWO SCHEDULE

Animal Number	Code	Week 0 Immunization	Week 4 Boost	Week 5 Test Bleed	Week 8 Boost	Week 9 Exsanguinate
204, 205	FBS1	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	16–Feb–94
101*	FBS1	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	18–Feb–94
206–208	FBS2A	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	16–Feb–94
209–211	FBS3	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	16–Feb–94
212–214	FBS4	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	16–Feb–94
215	FLIP	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	16–Feb–94
216	FLIP	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	17–Feb–94
102*	FLIP	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	18–Feb–94
217,218	CHE1	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	17–Feb–94
103*	CHE1	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	18–Feb–94
219–221	CHE2	14–Dec–93	11–Jan–94	18–Jan–94	08–Feb–94	17–Feb–94
222–224	CHE3	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	17–Feb–94
225–227	CHE4	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	17–Feb–94
228–230	CHE5	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	18–Feb–94
104*,231,232	CD41	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	18–Feb–94
233–235	CD42	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	18–Feb–94
236–238	CD43	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	18–Feb–94
239–241	KLH	15–Dec–94	12–Jan–94	19–Jan–94	09–Feb–94	18–Feb–94

* Rabbits previously used in the E–BChE Clearance Study, Protocol 141.

TABLE 6

FAB FRAGMENT ANTIBODIES SCHEDULE FOR RABBIT NOS. 401–406

WEEK	PROCEDURE	DATE
0	Pre–bleed and primary immunization	19–Oct–94
4	First boost immunization	16–Nov–94
5	Test Bleed	22–Nov–94
8	Second boost immunization	14–Dec–94
9	Test Bleed	21–Dec–94
11	Exsanguinate	04–Jan–95

196,2172–F\72–FT6

TABLE 7

REPEATED BChE SOLUTION OR MICROSPHERE INJECTION SCHEDULE FOR RABBIT NOS. 437–442

INJECTIONS/DATE		BLOOD DRAWING/DATE	
Day 1	2/27/95	Day 1 Pre–injection	2/27/95
		1 Hour	2/27/95
		4 Hour	2/27/95
		6–8 Hours	2/27/95
		Day 2	2/28/95
		Day 3	3/1/95
		Day 4	3/2/95
		Day 5	3/3/95
Day 8	3/6/95	Day 8 Pre–injection	3/6/95
		Day 9	3/7/95
		Day 12	3/10/95
Day 15	3/13/95	Day 15 Pre–injection	3/13/95
		Day 16	3/14/95
		Day 19	3/17/95
		Day 22	3/20/95
		Day 26	3/24/95
		Day 29	3/27/95
		Day 43	4/10/95

TABLE 8A

BLOOD LEVEL OF BUTYRYLCHOLINESTERASE ENZYME

FOLLOWING IV BOLUS INJECTION IN RABBITS

Time	Blood BChE, U/ml	
	Rabbit #101	Rabbit #102
Control	0.07	0.24
1 Hour	15.68	18.34
2 Hour	14.37	13.19
4 Hour	14.41	13.94
7 Hour	11.12	12.54
24 Hour	9.98	9.49
48 Hour	7.84	6.68
72 Hour	6.34	5.82
96 Hour	6.03	4.93
168 Hour	0.80	0.72

196.2172-F\72-FT8A

TABLE 8B

BLOOD LEVEL OF BUTYRYL CHOLINESTERASE ENZYME

FOLLOWING I.M. BOLUS INJECTION IN RABBITS

Time	Blood BChE, U/ml	
	Rabbit #103	Rabbit #104
Control	0.27	0.44
1 Hour	0.37	0.61
2 Hour	0.38	0.69
4 Hour	0.73	1.27
7 Hour	1.09	1.39
24 Hour	3.60	2.93
48 Hour	4.34	5.45
72 Hour	4.04	5.25
96 Hour	3.89	4.35
168 Hour	1.33	2.63

TABLE 9

PHARMACOKINETIC PARAMETERS AFTER INTRAVENOUS BOLUS

INJECTION OF HORSE SERUM BUTYRYL CHOLINESTERASE IN RABBITS

	AVERAGE (N=2)
DOSE (U/kg)	515
RABBIT WEIGHT (kg)	3.05
PARAMETER	
A (U/ml)	231.99
B (U/ml)	13.70
Alpha (per hr)	4.20
Beta (per hr)	0.01142
AVC	1252.58
Cmax (U/ml)	245.7
Volume	2.096
K 21 (per hr)	0.25
K 10 (per hr)	0.20
K 12 (per hr)	3.77
K 10−HL (hr)	3.53
Alpha−HL (hr)	0.16
Beta−HL (hr)	60.58
r^2	0.985

196,2172−F\72−F8T9

TABLE 10

PHARMACOKINETIC PARAMETERS AFTER INTRAMUSCULAR

INJECTION OF HORSE SERUM BUTYRYL CHOLINESTERASE IN RABBITS

	AVERAGE (N=2)
DOSE (U/kg)	527
RABBIT WEIGHT (kg)	2.98
PARAMETER	
A (U/ml)	17.655243
B (U/ml)	27.12
C= −(A+B)	−44.775942
Alpha (per hr)	0.034891
Beta (per hr)	0.014456
K 01	0.027177
Tmax (hr)	58.423
Cmax (U/ml)	4.80267
Volume	0.545361
K 21	0.048222
K 01	0.027177
K 10	0.010460
K 12	−0.009333
K 01−HL	25.51
K 10−HL (hr)	66.27
Alpha−HL (hr)	19.87
Beta−HL (hr)	47.95
r^2	0.997

196,2172−F\72−FT10

TABLE 11

ACHE and BChE RABBIT WEIGHTS

SAMPLE	STUDY	RABBIT NO.	WEIGHT (kg) 5/22/93	WEIGHT (kg) 6/7/93	WEIGHT (kg) 8/10/93	WEIGHT (kg) 11/2/94	WEIGHT (kg) 2/13/95
BChE	Long Term	96	2.80	2.95	3.50	4.05	4.00
BChE	Long Term	97	2.82	3.00	3.45	4.30	4.20
BChE	Long Term	98	2.70	3.05	3.67	4.35	4.10
BChE	Long Term	99	3.00	3.23	3.92	4.35	4.25
BChE	Long Term	100	2.60	2.95	3.60	4.55	4.55
AChE	Long Term	105	3.00	3.32	3.77	4.60	4.65
AChE	Long Term	106	2.65	2.93	3.55	4.25	4.15
AChE	Long Term	107	2.80	2.95	3.50	3.80	3.85
AChE	Long Term	108	2.80	3.05	3.80	4.85	4.95
AChE	Long Term	109	2.67	2.92	3.50	4.40	3.35

196.2172-F\72-FT11

TABLE 12

PEPTIDE RABBIT WEIGHTS (kg's)

SAMPLE	RABBIT NO.	WEIGHT (kg) 4/19/93	WEIGHT (kg) 6/7/93	WEIGHT (kg) 8/10/93
XNAT	59	2.55	3.20	3.77
XNAT	60	2.63	3.25	3.90
XRDA	61	2.60	3.50	3.95
XRDA	62	2.90	3.85	4.57
XCNB	63	2.85	3.63	4.30
XCNB	64	2.70	3.50	3.92
SCD4	65	2.85	3.40	3.80*
SCD4	66	2.85	3.52	3.95*
CD41	67	2.72	3.45	3.80
CD41	68	2.93	3.60	3.95
CD42	69	2.90	3.45	3.90
CD42	70	2.90	3.50	3.90
CD43	71	2.80	3.55	3.84
CD43	72	2.65	3.40	3.90
FLIP	73	2.80	3.55	3.95
FLIP	74	2.70	3.35	3.90
XN8	75	2.50	3.15	3.72
XN8	76	2.40	3.00	3.50
NTR1	77	2.65	3.48	3.95
NTR1	78	2.35	3.22	3.68
NTR2	79	2.60	3.40	3.92
NTR2	80	2.40	3.00	3.70
NTR3	81	2.50	3.30	3.75
NTR3	82	2.48	3.32	3.80
NTR4	83	2.70	3.62	4.25
NTR4	84	2.75	3.40	3.85
NTR5	85	2.50	3.22	3.78
NTR5	86	2.80	3.63	4.00

* Weight on 7/13/93

TABLE 13

PEPTIDE STUDY TWO RABBIT WEIGHTS

Animal Number	Sample Code	12-06-93 Wt. kg
101	FBS1	4.15
102	FLIP	3.90
103	CHE1	4.00
104	CD41	4.20
204	FBS1	2.70
205	FBS1	2.65
206	FBS2A	2.60
207	FBS2A	2.70
208	FBS2A	2.55
209	FBS3	2.70
210	FBS3	2.75
211	FBS3	2.80
212	FBS4	2.80
213	FBS4	2.50
214	FBS4	2.60
215	FLIP	2.55
216	FLIP	2.90
217	CHE1	2.95
218	CHE1	2.73
219	CHE2	2.60
220	CHE2	2.55

Animal Number	Sample Code	12-06-93 Wt. kg
221	CHE2	2.50
222	CHE3	2.58
223	CHE3	2.60
224	CHE3	2.65
225	CHE4	2.60
226	CHE4	2.70
227	CHE4	2.58
228	CHE5	2.50
229	CHE5	2.55
230	CHE5	2.50
231	CD41	2.55
232	CD41	2.75
233	CD42	2.60
234	CD42	2.65
235	CD42	2.58
236	CD43	2.45
237	CD43	2.40
238	CD43	2.55
239	KLH	2.65
240	KLH	2.70
241	KLH	2.65

196,2172-F\72-FT13

TABLE 14

FAB FRAGMENT ANTIBODY RABBIT WEIGHTS

SAMPLE	STUDY	RABBIT NO.	WEIGHT (kg) 10/18/94	WEIGHT (kg) 11/2/94	WEIGHT (kg) 12/22/94	WEIGHT (kg) 1/4/95
E4–13D8	Antibody	401	2.60	2.72	3.25	3.45
E4–13D8	Antibody	402	2.48	2.60	3.05	3.20
E4–13D8	Antibody	403	2.65	2.80	3.35	3.40
E5–25B1	Antibody	404	2.20	2.30	2.95	3.00
E5–25B1	Antibody	405	2.45	2.55	3.10	3.20
E5–25B1	Antibody	406	2.60	2.80	3.30	3.50

196,2172–F\72–FT14

TABLE 15

W/O MICROSPHERE CORE PREPARATIONS

Sample Name	Process Conditions		Starting Dry Components (grams/%total)							Liquid Components		Analysis Performed									
	Continuous Phase	RPM's/ # baffles	$CaSO_4$	Hydroxy-apatite	RSA	BSA	BChE 8.7 U/mg	BChE 610 U/mg	Lac-tose	Distilled water	Misc.	% of expected content Protein	% of expected content Activity	% released Protein	% released Activity	% released Ca	Duration of release (days) Protein	Duration of release (days) Activity	Duration of release (days) Ca	% in 105–150 µm	SEM
88MS15	vegetable	2000	10				1			8 ml		57%	55%	100%	34%		42	5			yes
	oil	1	90.9%				9.1%														
88MS17	vegetable	2000	9.8				0.2			8 ml		54%	60%	55%	0		15	0			
	oil		98.0%				2.0%														
88MS19	vegetable	1200	2.5					0.009		2.5 ml		0%	0%			112%			4		yes
	oil		99.6%					0.4%													
88MS20	vegetable	1200	2.5					0.009		2.5 ml		90%	0%								
	oil		99.6%					0.4%													
88MS21	vegetable	2000	9				0.9			8 ml		81%	67%								
	oil		90.9%				9.1%														
88MS22	vegetable	2000	1.14	1.14			0.25			9 ml											
	oil		45.1%	45.1%			9.9%					1.9%							20		
72MS8	vegetable	2000/1200	10				1			8 ml							3				
	oil	1	90.9%				9.1%														
72MS9	vegetable	2000/1200	10							1 ml							8				
	oil	1	100.0%																		
72MS10	vegetable	2450/700	11				1			8 ml											yes
	oil	1	91.7%				8.3%													19.7	
72MS14	vegetable	2000/1200	2.25			0.25		0.003		1 ml			16%								
	oil	0	89.9%			10.0%		0.1%													
72MS15	vegetable	2000/1200	2.25			0.25		0.003		1 ml			>20%								yes
	oil	0	89.9%			10.0%		0.1%													
72MS16	vegetable	2000/1200	2.25					0.003	0.25	1 ml			<5%								yes
	oil	0	89.9%					0.1%	10.0%						19%						
72MS28	vegetable	1650	3.68					0.32		9 ml	1 ml										
	oil	0	92.0%					8.0%			Tween 80									1.1	
72MS29	vegetable	2000	3.68					0.32		9 ml	1 ml										
	oil	0	92.0%					8.0%			Tween 20									6.2	
72MS30	vegetable	2000	3.97				0.003			10 ml	1 gm										
	oil	0	99.9%				0.1%				Mannitol									17.9	
72MSG1	vegetable	2200/1200	2.55			0.24	none	none		1.9 ml	.1 ml										
	oil		91.4%			8.6%					glycerol										
72MSG2	vegetable	1750/650	2.25		0.196			0.05		1.98 ml	.02 ml										yes
	oil	0	90.1%		7.9%			2.0%			glycerol		25.8%								
72MSG3	vegetable	1850/650	2.25		0.202			0.05		2.145 ml	.055 ml		25.8%								
	oil	0	89.9%		8.1%			2.0%			glycerol										
72MSG4	vegetable	2100/1000	2.29		0.202		none	none		2.145 ml	.055 ml										
	oil	0	91.9%		8.1%						glycerol										
72MSG5	vegetable	2100/1000	2.28		0.2			0.045		2.145 ml	.055 ml										
	oil	0	90.3%		7.9%			1.8%			glycerol		25.8%								
72MSG6	vegetable	1800/1000	9.2			0.8	none	none		6.435 ml	0.165 ml					100%			2		
scale-up	oil	0	92.0%			8.0%					glycerol									11.4	
72MSG7	vegetable	2000/1000	9.2			0.8	none	none		6.435 ml	0.165 ml					100%			2		
scale-up	oil	0	92.0%			8.0%					glycerol									14.4	
72MSG8	vegetable	2000/1000	9.2			0.8	none	none		6.435 ml	0.165 ml					100%			2		
scale-up	oil	1	92.0%			8.0%					glycerol									11.3	
72MSG9	vegetable	2200/1000	9.2			0.8	none	none		6.435 ml	0.165 ml					100%			2		
scale-up	oil	0	92.0%			8.0%					glycerol									9.6	
72MSG10	vegetable	2000/1000	9.2			0.8	none	none		6.435 ml	0.165 ml					100%			2		
scale-up	oil	0	92.0%			8.0%					glycerol									12.0	
72MSG11	vegetable	2000/1000	9.2			0.8	none	none		5.94 ml	0.66 ml										
scale-up	oil	0	92.0%			8.0%					glycerol									9.1	
72MSG12	vegetable	2000/1000	9.2			0.8	none	none		5.94 ml	0.66 ml										
scale-up	oil	0	92.0%			8.0%					glycerol									20.3	
72MSG13	vegetable	2000/1000	9.2			0.8	none	none		5.94 ml	0.66 ml										
scale-up	oil	0	92.0%			8.0%					glycerol									10.2	
72MSG14	vegetable	2000/1000	9.2			0.8	none	none		5.94 ml	0.66 ml										
scale-up	oil	0	92.0%			8.0%					glycerol										
72MSG15	vegetable	1500/1200	9.2				0.8			7.2 ml	0.8 gm										
scale-up	oil	1	92.0%				8.0%				mannitol									37.7	

196,2172-F\72-FT15

TABLE 16

CALCIUM SULFATE MICROCAPSULES PREPARED BY OVERCOATING

Sample Name	Core Conditions		Pan Coating		Process Conditions				Analysis Performed								
									% of Expected Content		% Released			Duration of Release (days)			SEM
	Core Material	Core Size Range	Triglyceride Type	Theoretical Coating Level	Polymer Type	Polymer Solvent	Soln Conc	Theoretical Coating Level	Protein	Activity	Protein	Activity	Ca	Protein	Activity	Ca	
72W1	72MS8	105–150	Suppocire–D	10%	75/25 PDLLA–PGA	Acetone	1%	10%	96.7%		92%			42			yes
72W2	72MS9	25–250	Suppocire–D	10%	75/25 PDLLA–PGA	Acetone	1%	10%						5			
72W3	72MS10	53–180	Suppocire–D	10%	75/25 PDLLA–PGA	Acetone	1%	10%						35			
72W4	72MSB1*	75–150	none		65/35 PLLA–PGA	Acetone	1%	10%					97.2%			149	
88W1	88MS15	53–250	Suppocire–D	10%	75/25 PDLLA–PGA	Acetone	1%	15%	62%	42%	100%	15%	107.9%	49	5	57	yes
88W2	88MS17	53–250	Suppocire–D	10%	75/25 PDLLA–PGA	Acetone	1%	20%	40%	1%	50%	0%		21			
88W3	88MS21	53–250	Suppocire–D	10%	75/25 PDLLA–PGA	Acetone	1%	15%	82%	45%			146%			8	

196,2172–F\72–FT16

* A blend of 72MSG6 through 72MSG10

TABLE 17

W/O/W MICROSPHERE CORE PREPARATIONS

Sample Name	Process Conditions: Continuous Phase	Process Conditions: RPM's/ # baffles	Starting Dry Components (grams/%total): $CaSO_4$	$CaHPO_4$	Hydroxy-apatite	Polymer wt.	Polymer Type	Miscellaneous: Suppocire-D	Miscellaneous: Dynasan	BSA	BChE: 6.7 U/mg	BChE: 610 U/mg	Liquid Components: Distilled water	Liquid Components: Misc.	Analysis Performed: % of expected content: Protein	% of expected content: Activity	% released: Protein	% released: Activity	% released: Ca	Duration of release (days): Protein	Duration of release (days): Activity	Duration of release (days): Ca	% in 105–150 µm	SEM
72MS1	water	710/510				2	65/35 L-					0.200	1 ml	10 ml $MeCl_2$			100			2				
	1% PVA (RT)	2				90.9%						9.1%		5 ml EtAc									13.25	
72MS2	water	1160/950/500				5	75/25					0.080	1 ml	9 ml						6				
	1% PVA (RT)	2				98.4%	PLGA					1.6%		$MeCl_2$									12.99	
72MS3	water	1150/950/500				5	65/35 L-					0.084	1 ml	9 ml						7				
	1% PVA (RT)	2				98.3%						1.7%		$MeCl_2$										
72MS4	water	1100/850/500				4.95	65/35 L-					0.05	1 ml	9 ml										
	1% PVA (RT)	2				99.0%						1.0%		$MeCl_2$									9.6	
72MS5	water	850/600				2.05	85/15		0.15			0.027	1 ml	9 ml						1				
	1% PVA (RT)	2				92.1%	PLGA		6.7%			1.2%		$MeCl_2$									37.2	
72MS6	water	850/600				2.06	85/15		0.13			0.19	1 ml	9 ml						6				
	1% PVA (RT)	2				86.7%	PLGA		5.4%			7.9%		$MeCl_2$									27.08	
72MS7	water	850/600				1	50/50					0.103	1 ml	9 ml						38				
	1% PVA (RT)	2				90.7%	PLGA					9.3%		$MeCl_2$										
72MS17	water	650/450	2.49			1	65/35 L-			0.056		0.42	4 ml	20 ml						30	1			yes
	1% PVA (RT)	2	62.8%			25.2%				1.4%		10.6%		$MeCl_2$	12%	25%		20%					29.73	
72MS18	water	550/450	2.25			1	65/35 L-			0.25	0.003		2.2 ml	20 ml						23	1	2		yes
	1% PVA (RT)	2	64.2%			28.5%				7.1%	0.1%			$MeCl_2$	46%	90%		3%	70.2%				39.37	
72MS19	water	575/450	2.5			1	65/35 L-					0.5	2 ml	20 ml						18		2		yes
	1% PVA (RT)	2	62.5%			25.0%						12.5%		$MeCl_2$	30%	68%		12%	75.8%				49.57	
72MS20	water	575/450	10.06			4.01	65/35 L-					0.879	8 ml	80 ml						18		2		yes
	1% PVA (RT)	2	67.3%			26.8%						5.9%		$MeCl_2$	2%	0%		2%	1.4%				53.25	
72MS21	water	575/450	2.25					1		0.25			2.2 ml	20 ml								12		yes
	1% PVA (RT)	0	64.3%					28.6%		7.1%				$MeCl_2$	3%				7.2%				50	
72MS22	water	575/450	2.25			1	65/35 L-	1		0.25			2.2 ml	20 ml								5		yes
	1% PVA (RT)	0	50.0%			22.2%		22.2%		5.6%				$MeCl_2$	50%				63.2%				23.3	
72MS23	water	575/450	2.25			0.5	65/35 L-			0.25			2.2 ml	20 ml								2		yes
	1% PVA (RT)	0	75.0%			16.7%				8.3%				$MeCl_2$	99%				53.6%				7.59	
72MS24	water	575/450	2.25			1	50/50			0.25			2.2 ml	20 ml								2		yes
	1% PVA (RT)	0	64.3%			28.6%	PLGA			7.1%				$MeCl_2$	75%				54.1%				36.61	
72MS25	water	575/450				1	65/35 L-			0.25			2.2 ml	20 ml								2		yes
	1% PVA (RT)	0				80.0%				20.0%				$MeCl_2$	50%				0%				21.84	
72MS26	water	575/450	1.25	1.25		1	65/35 L-			0.25			2.2 ml	20 ml								49		yes
	1% PVA (RT)	0	33.3%	33.3%		26.7%				6.7%				$MeCl_2$	105%				60.4%				25.42	
72MS27	water	550/450	1.25		1.25	1	65/35 L-			0.25			2.2 ml	20 ml								21		yes
	1% PVA (RT)	2	33.3%		33.3%	26.7%				6.7%				$MeCl_2$	7%				27%				18.7	

196,2172-F\72-FT17

TABLE 18

REPEATED DOSE BUTYRYLCHOLINESTERASE MICROSPHERES

RABBIT WEIGHTS STUDY

SAMPLE	RABBIT NO.	WEIGHT (kg) 2/27/95	WEIGHT (kg) 3/27/95
BChE Enzyme	437	3.05	3.20
BChE Enzyme	438	3.05	3.25
BChE Enzyme	439	3.10	3.30
BChE Microsphere	440	3.30	3.45
BChE Microsphere	441	2.95	3.25
BChE Microsphere	442	3.00	3.20

196,2172–F\72–FT18

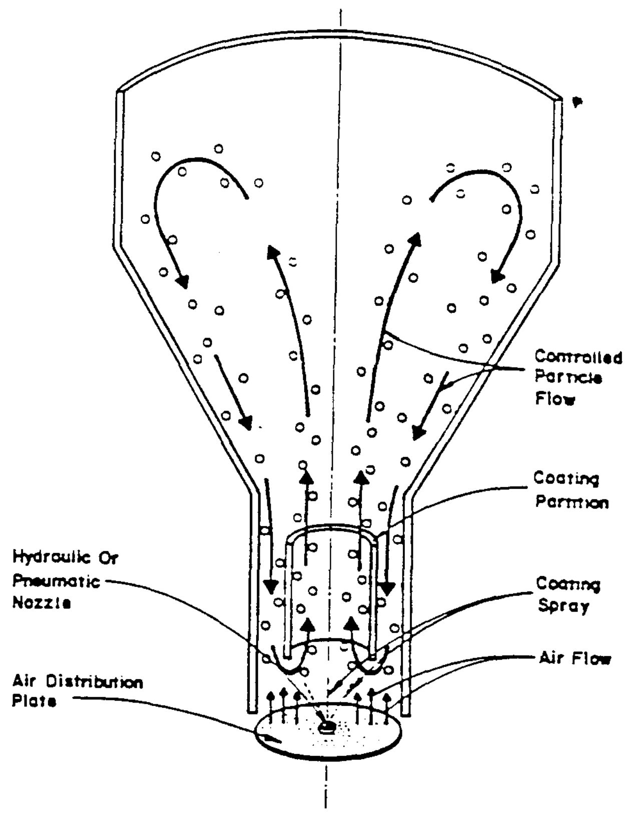


FIGURE 1 - Illustration of the Principle of Air Suspension Microencapsulation Process.

FIGURE 2

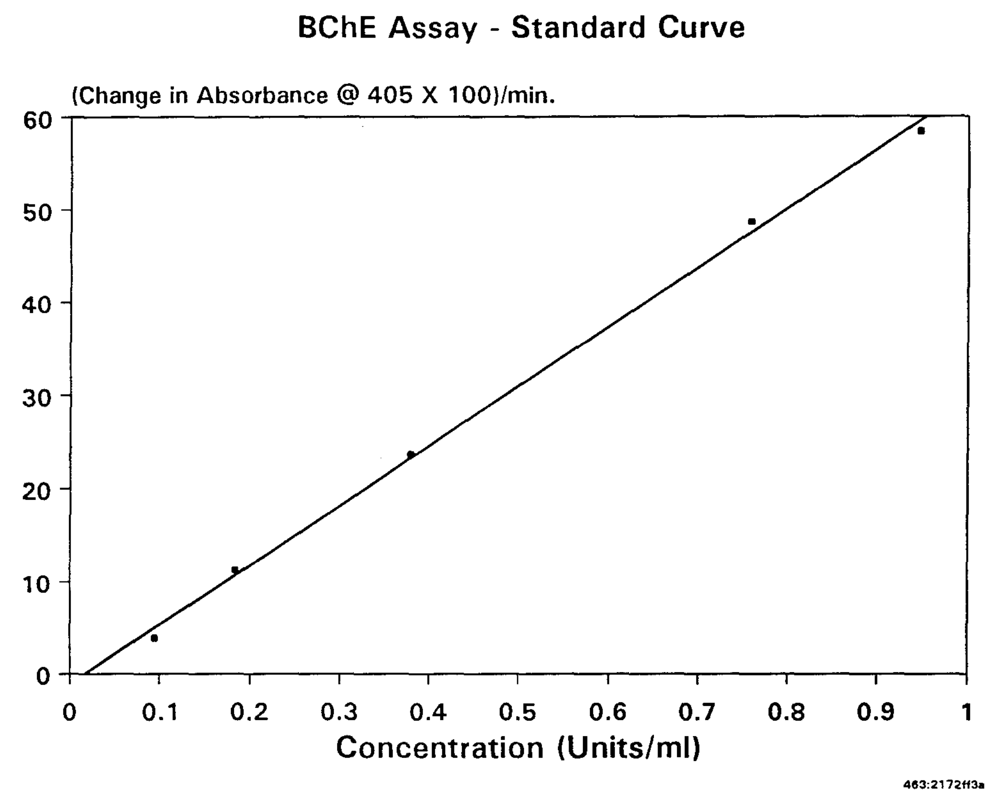


FIGURE 3

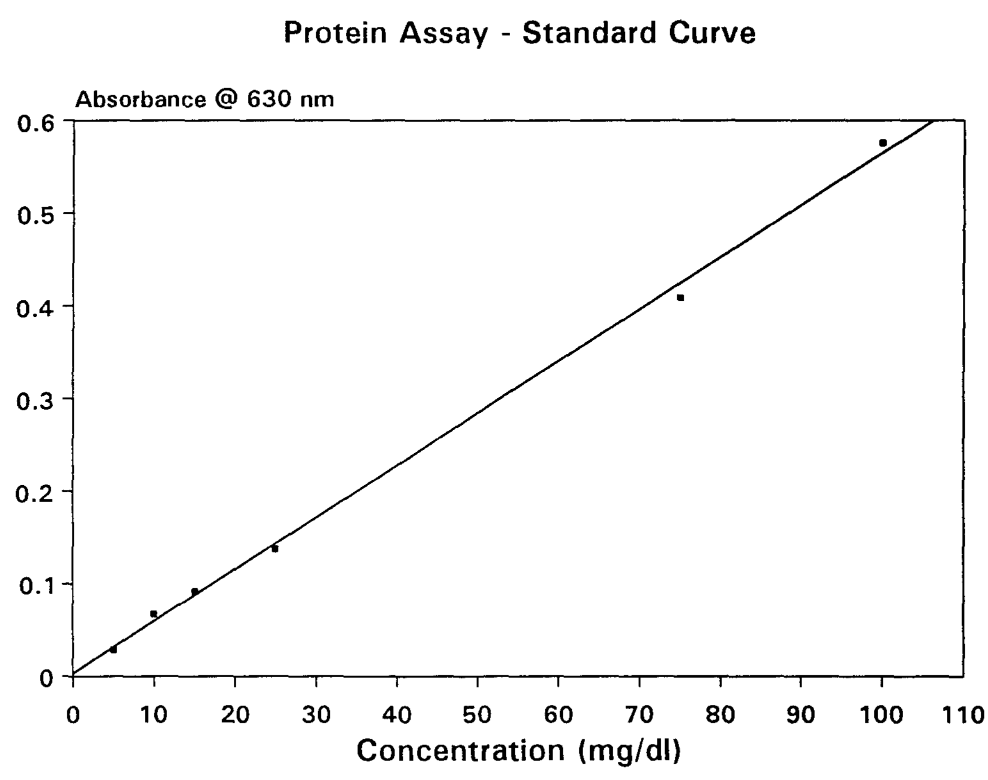
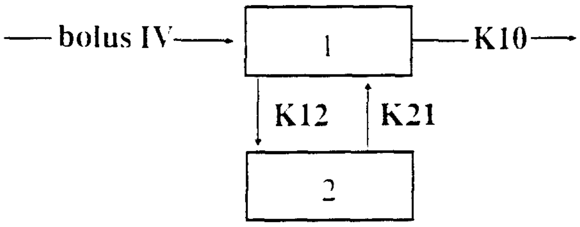


FIGURE 4

MODEL 8. Two-compartment with bolus input and first-order output; macro-constants as primary parameters.



C(T) = A*EXP(-ALPHA*T) + B*EXP(-BETA*T)

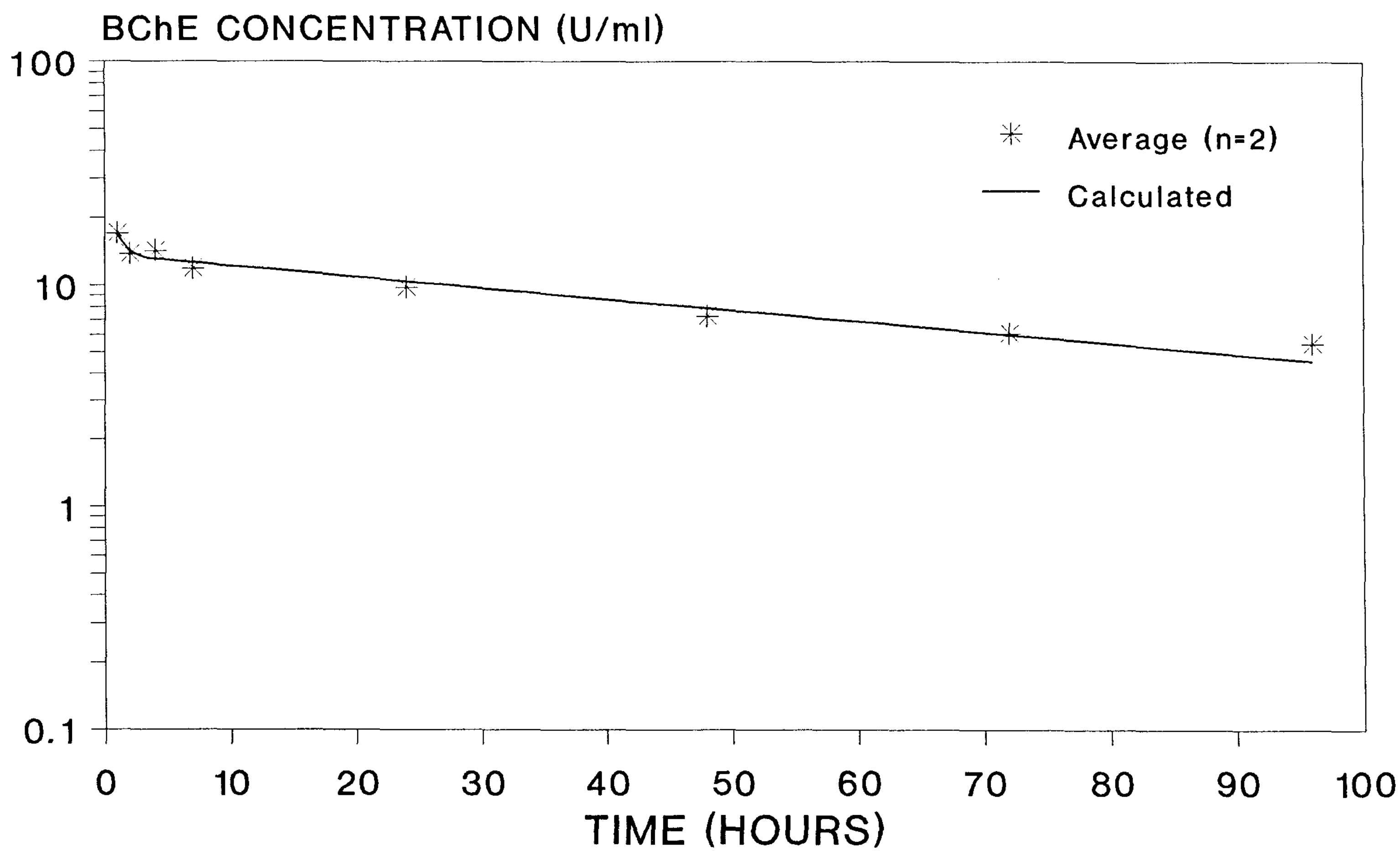
Estimated parameters:
(1) A
(2) B
(3) alpha
(4) beta

Constants in input:
(1) stripping dose
(2) # doses
(3) dose 1
(4) time of dose 1
(Repeat 3-4 for additional doses)

Secondary parameters:
(1) AUC = A/alpha + B/beta
(2) K10 half-life
(3) ALPHA half-life
(4) BETA half-life
(5) K10
(6) K12
(7) K21
(8) volume
(9) CMAX = D/V

FIGURE 5

IV CLEARANCE OF BChE* IN RABBITS
PCNONLIN Two Compartment Model 8
(Bolus Input & First Order Output)

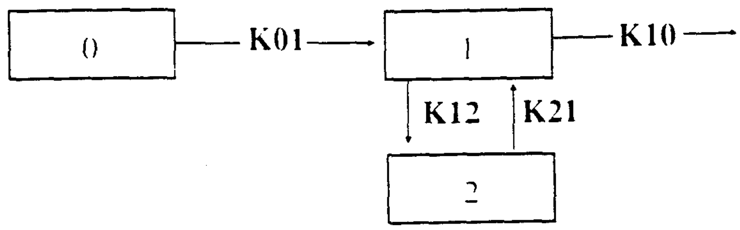


*Horse Serum BChE @ a Dose of 515 U/kg

HG/17/BCHE-IV

FIGURE 6

MODEL 13. Two-compartment with first-order input, first-order output, no lag time and macro-constants as primary parameters.



C(T) = A*EXP(-ALPHA*T) + B*EXP(-BETA*T) +
C*EXP(-K01*T).

Estimated parameters:
(1) A
(2) B
(3) K01 = absorption rate
(4) alpha
(5) beta
(NOTE: C = -(A + B))

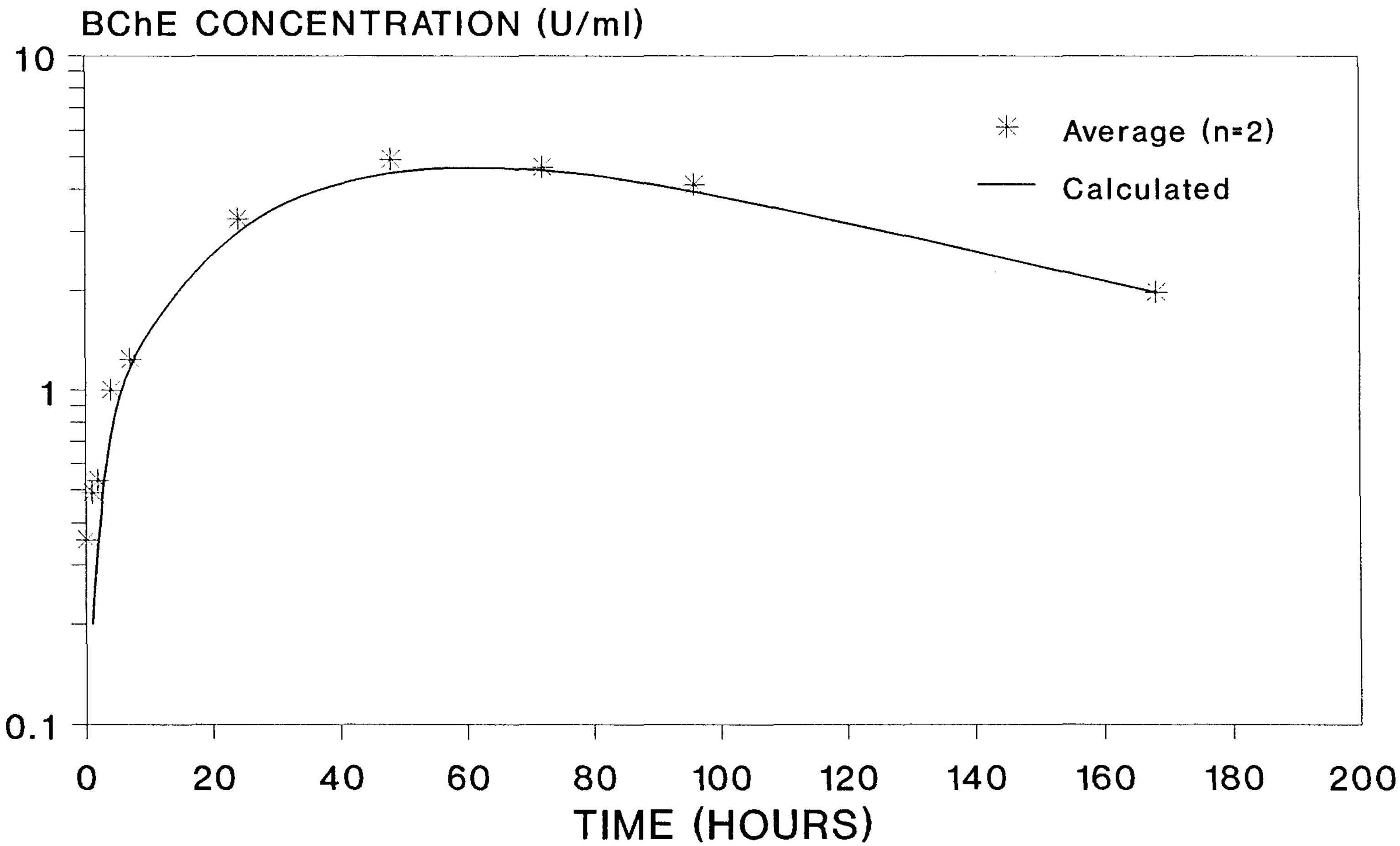
Constants in input:
(1) stripping dose
(2) # doses
(3) dose 1
(4) time of dose 1
(Repeat 3-4 for additional doses)

Secondary parameters:
(1) K10
(2) K12
(3) K21
(4) AUC = D/V/K10
(5) K10 half-life
(6) K01 half-life
(7) ALPHA half-life
(8) BETA half-life
(9) volume
(10) Tmax*
(11) Cmax*

* Estimated for the compiled (internal) library only.

FIGURE 7

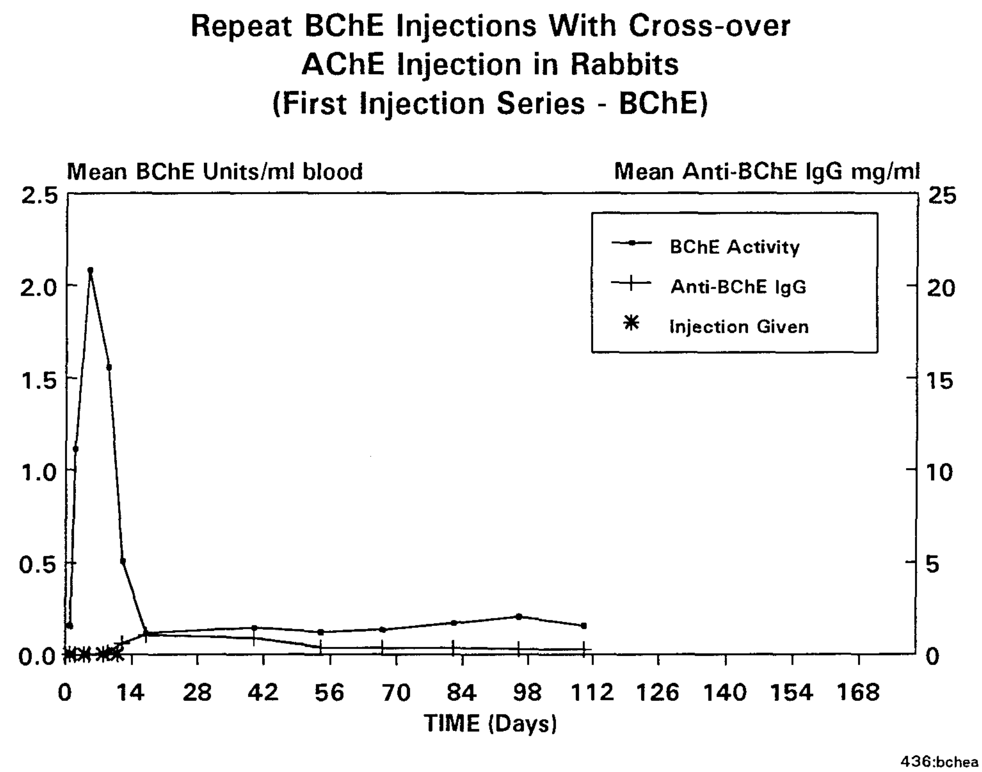
IM CLEARANCE OF BChE* IN RABBITS
PCNONLIN Two Compartment Model 13
(First Order Input & First Order Output)



*Horse Serum BChE @ a Dose of 527 U/kg

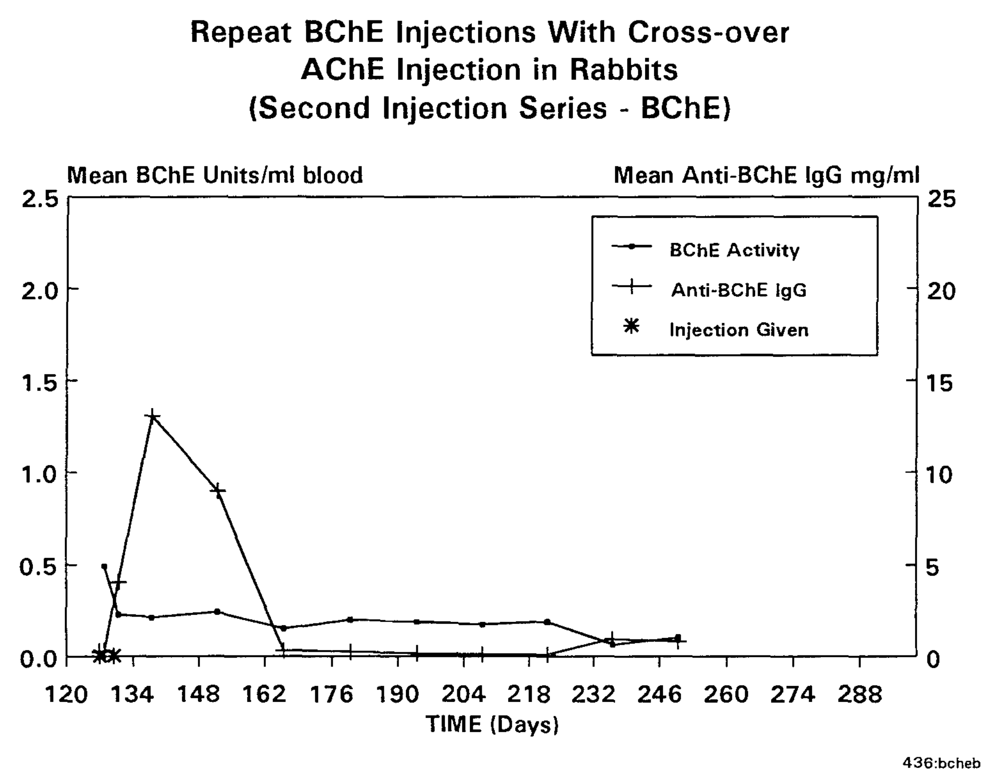
HG/17/BCHE-IM

FIGURE 8A



Data courtesy of Ms. M.K. Gentry, WRAIR

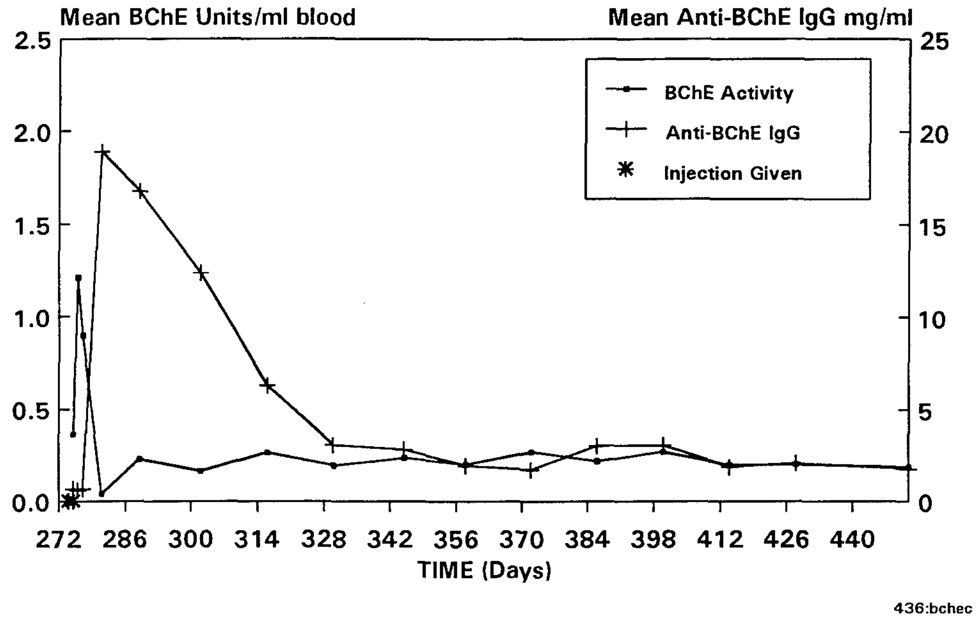
FIGURE 8B



Data courtesy of Ms. M.K.Gentry, WRAIR

FIGURE 8C

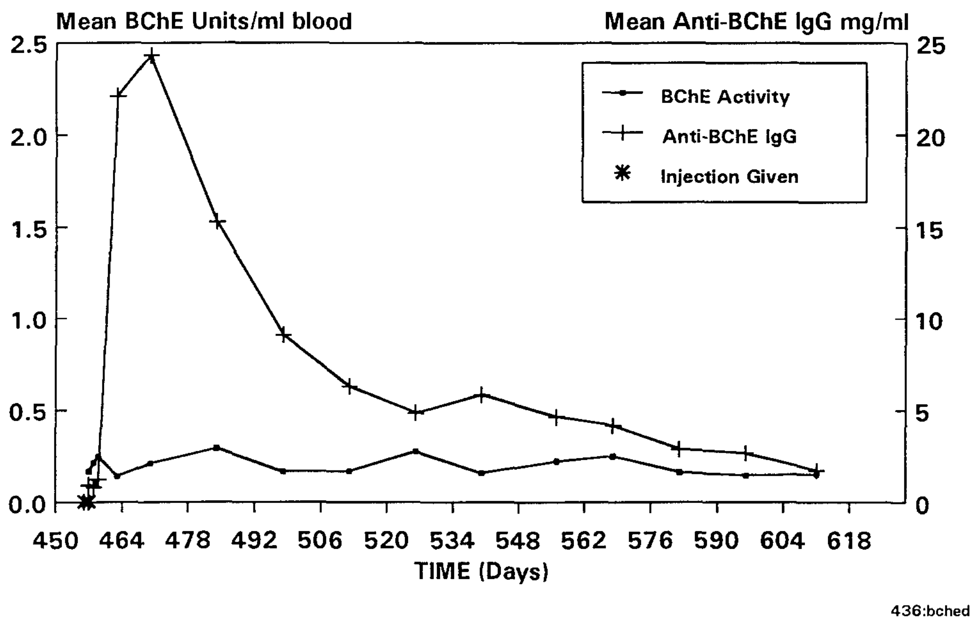
Repeat BChE Injections With Cross-over AChE Injection in Rabbits (Third Injection Series - BChE)



Data courtesy of Ms. M.K. Gentry, WRAIR

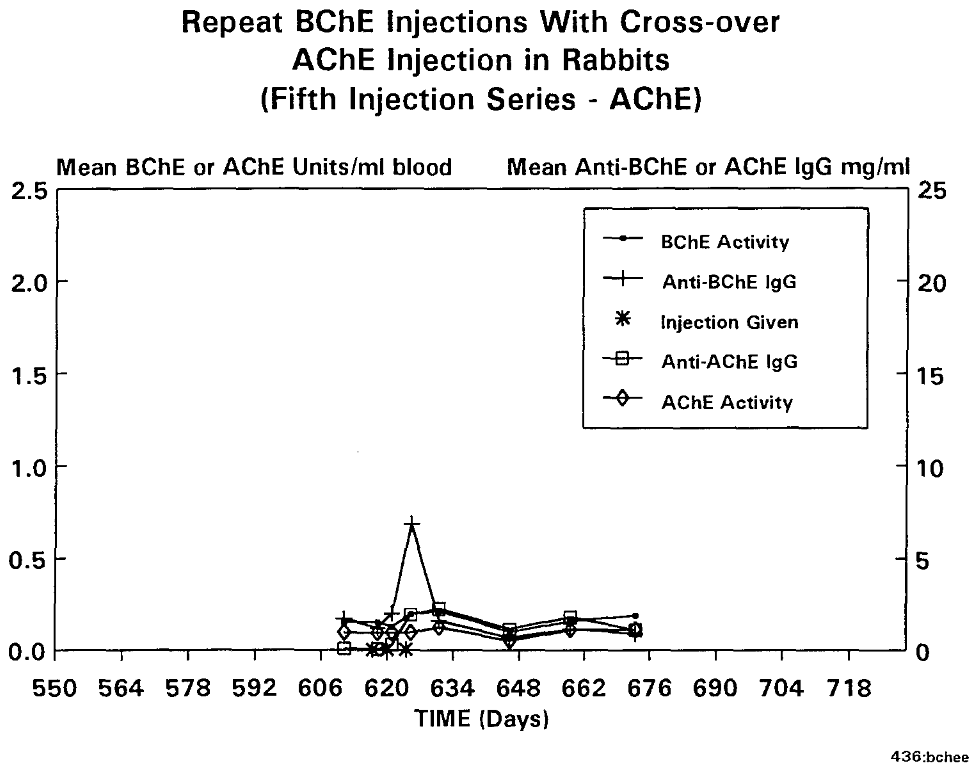
FIGURE 8D

Repeat BChE Injections With Cross-over AChE Injection in Rabbits (Fourth Injection Series - BChE)



Data courtesy of Ms. M.K. Gentry, WRAIR

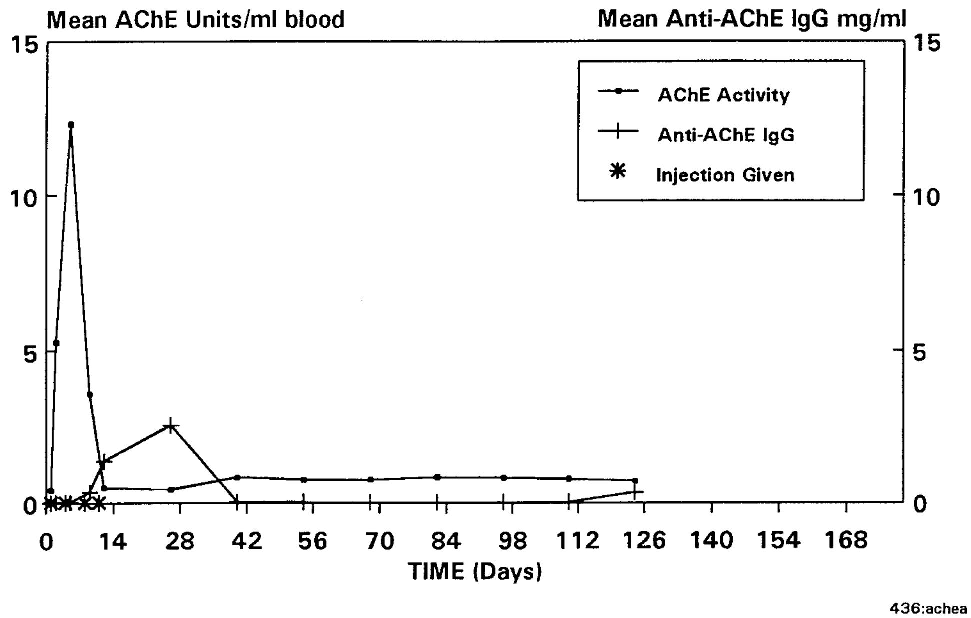
FIGURE 8E



Data courtesy of Ms. M.K. Gentry, WRAIR

FIGURE 9A

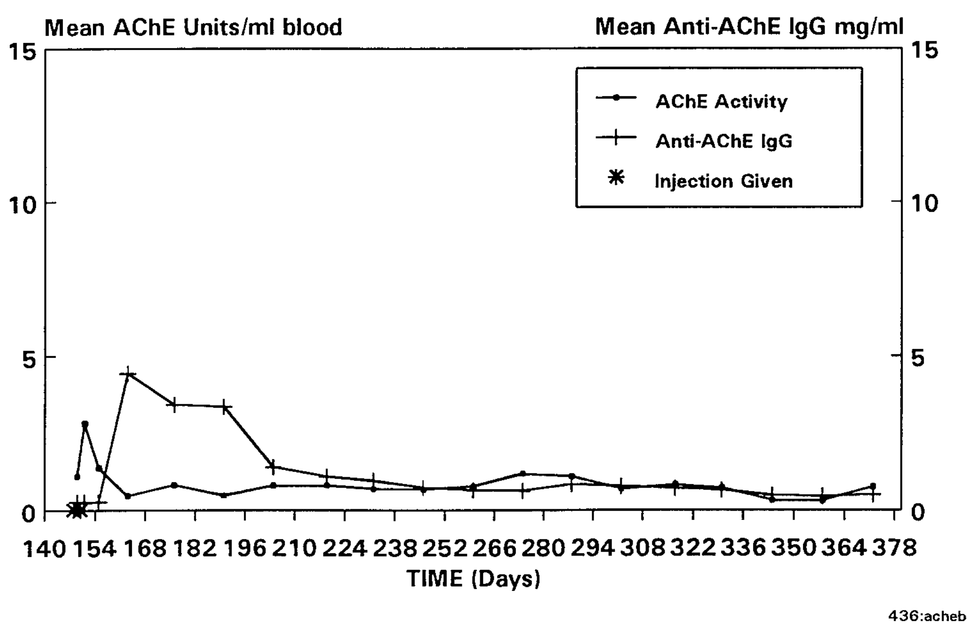
Repeat FBS-AChE Injections With Cross-over BChE Injection in Rabbits (First Injection Series - AChE)



Data courtesy of Ms. M.K. Gentry, WRAIR

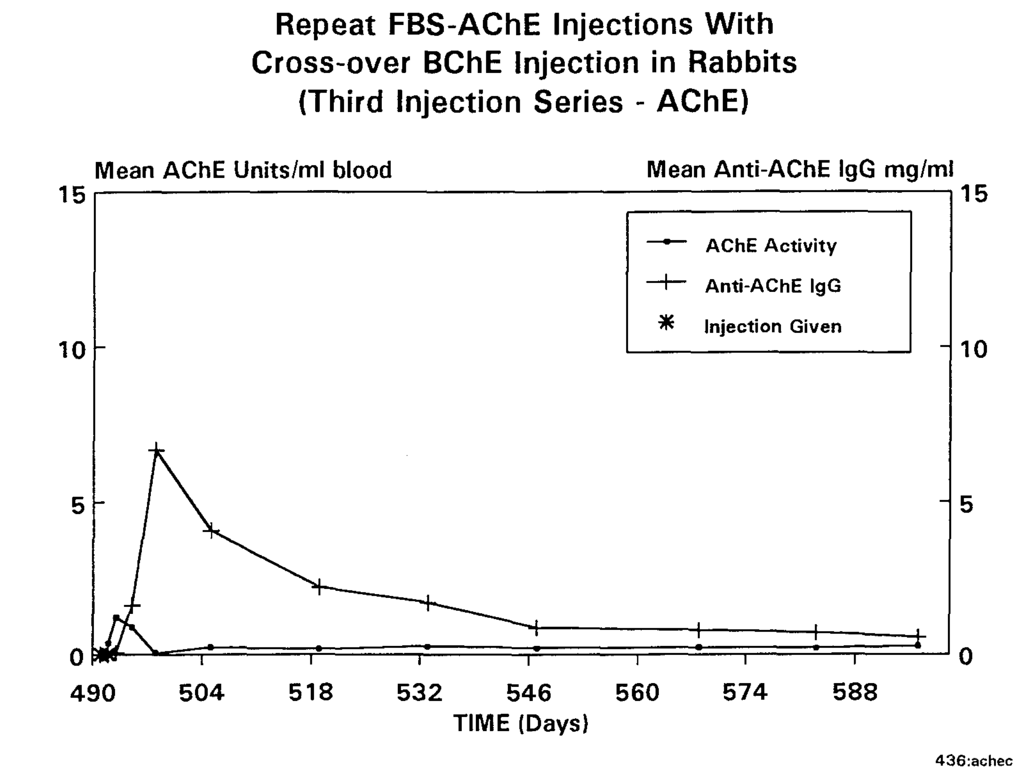
FIGURE 9B

Repeat FBS-AChE Injections With Cross-over BChE Injection in Rabbits (Second Injection Series - AChE)



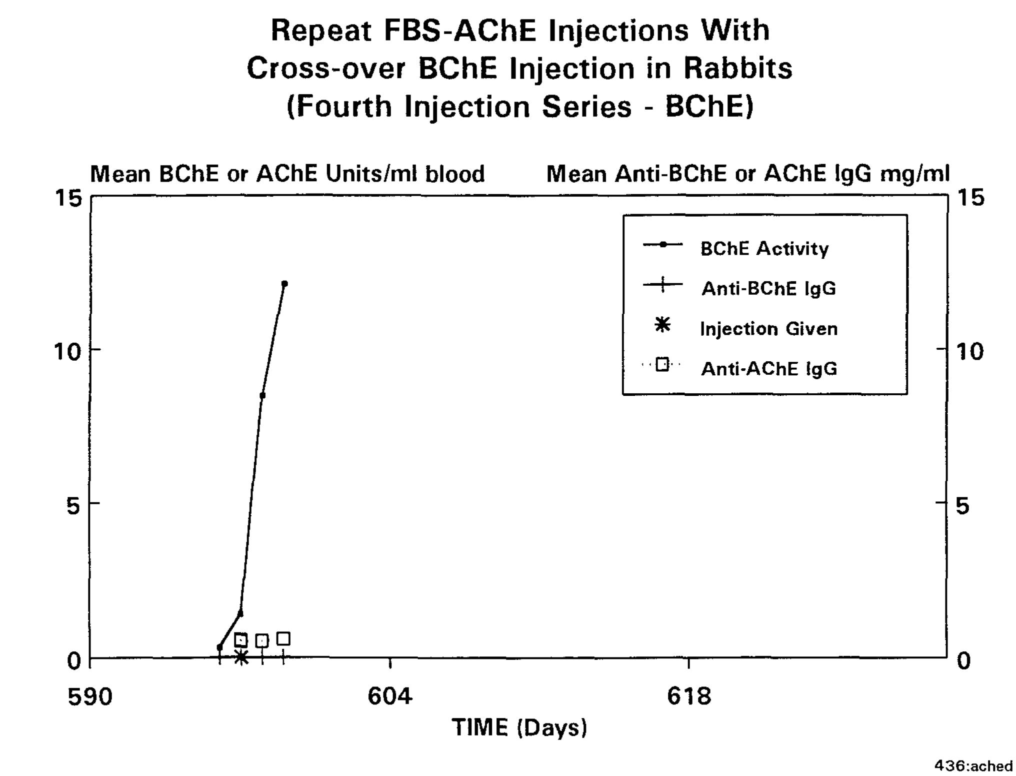
Data courtesy of Ms. M.K. Gentry, WRAIR

FIGURE 9C



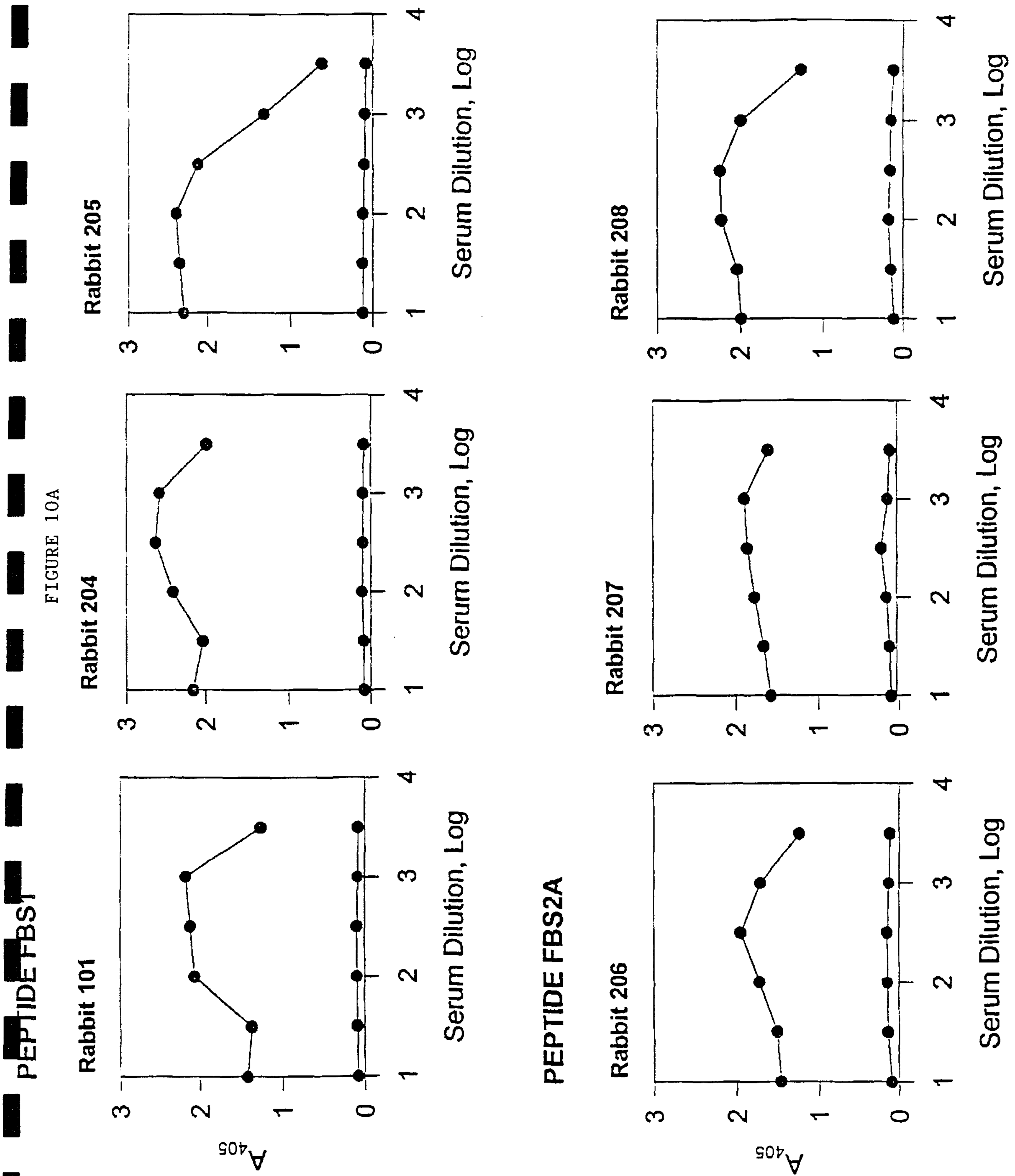
Data courtesy of Ms. M.K. Gentry, WRAIR

FIGURE 9D



Data courtesy of Ms. M.K. Gentry, WRAIR

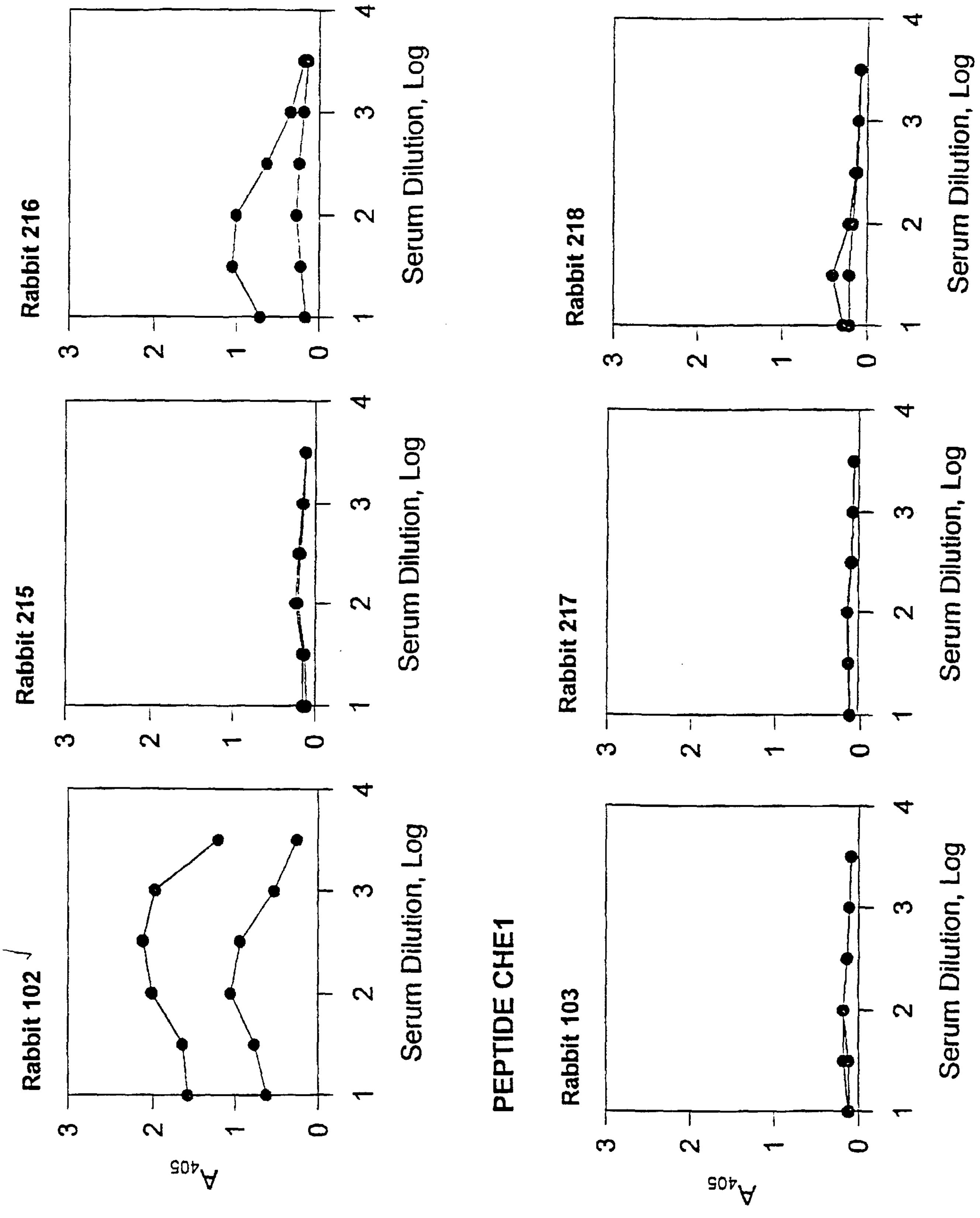
FIGURE 10A



Data courtesy of Ms. Mary Kay Gentry WRAIR

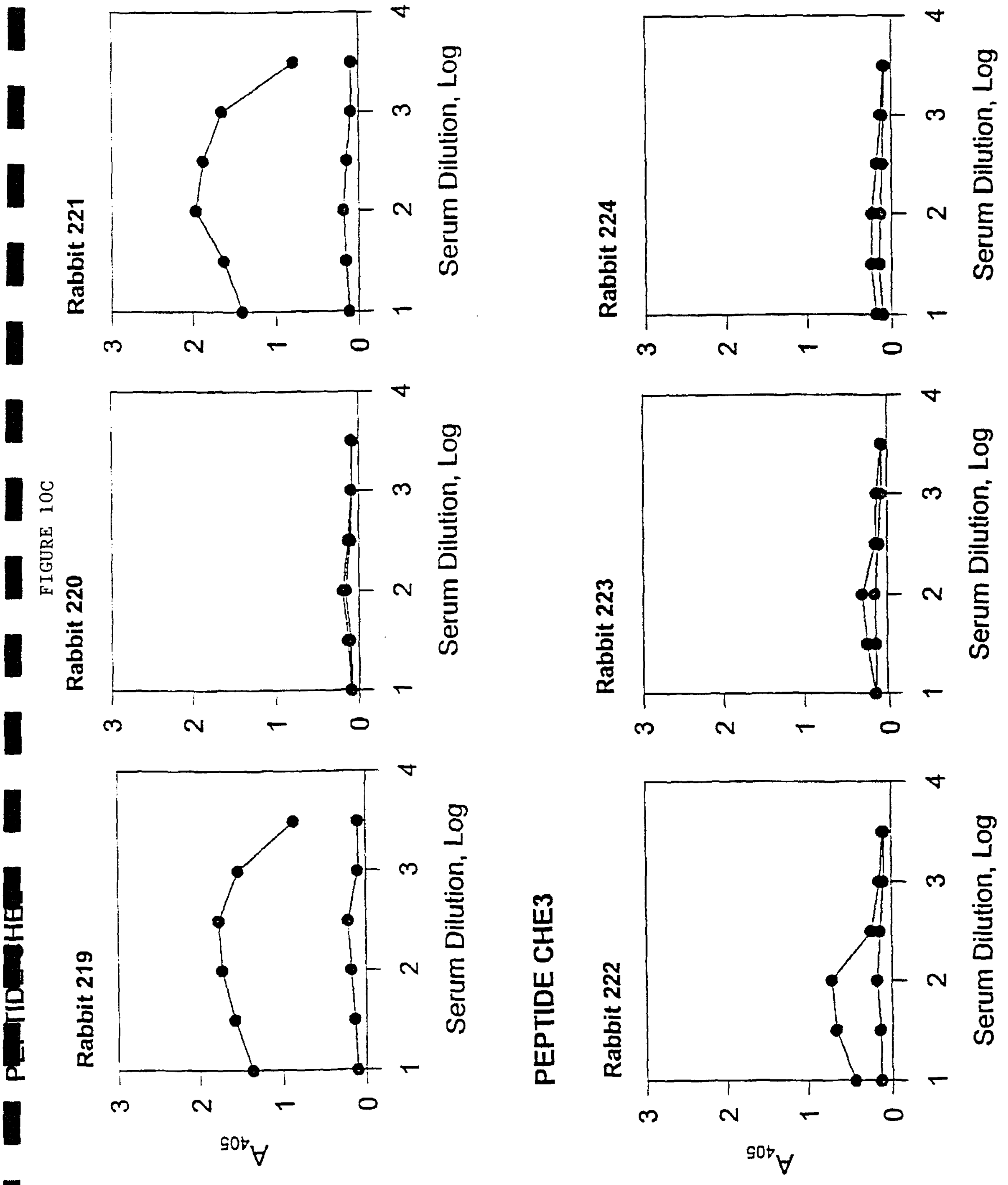
FIGURE 10B

P[redacted]TID[redacted]LIR



Data courtesy of Ms. Mary Kay Gentry WRAIR

FIGURE 10C



Data courtesy of Ms. Mary Kay Gentry WRAIR

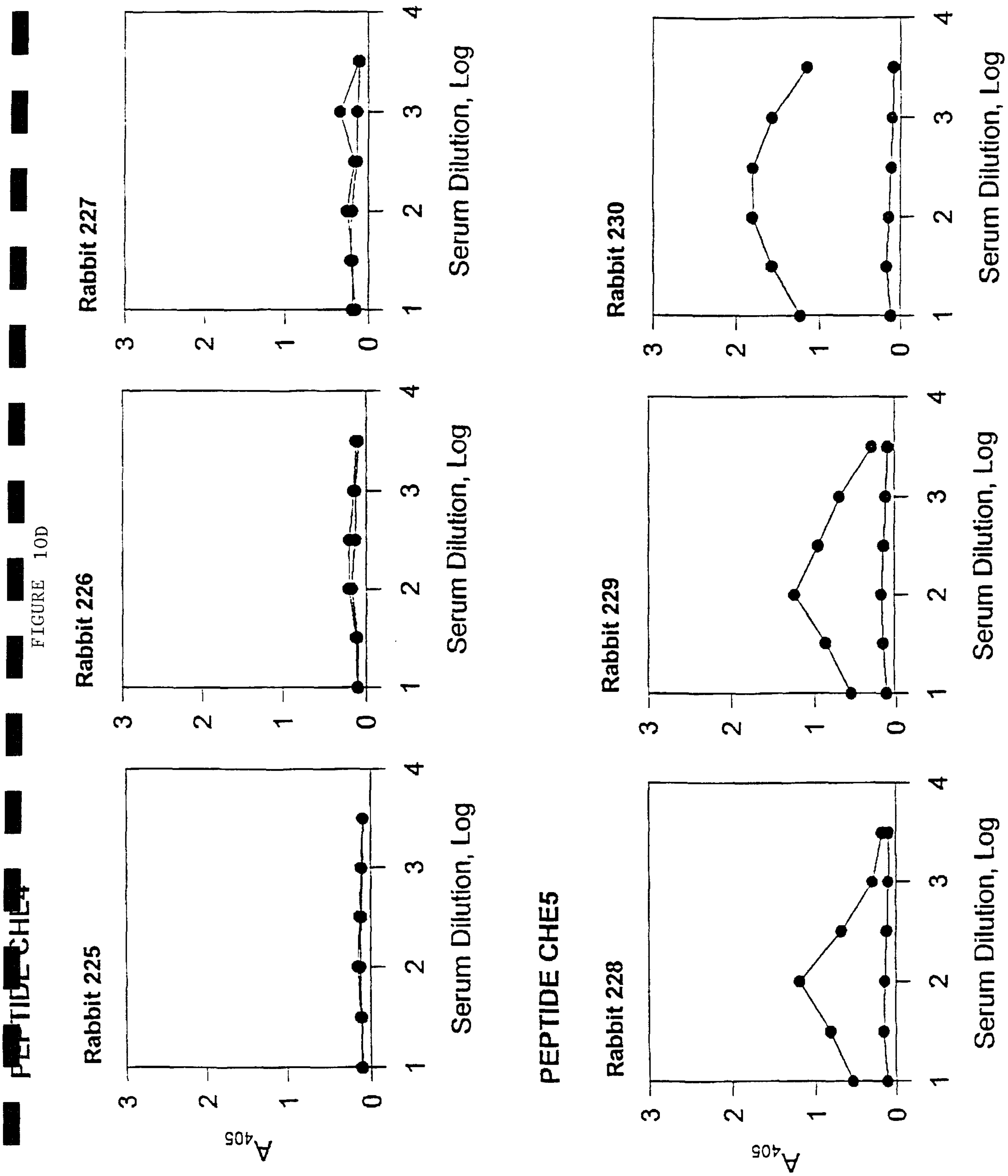
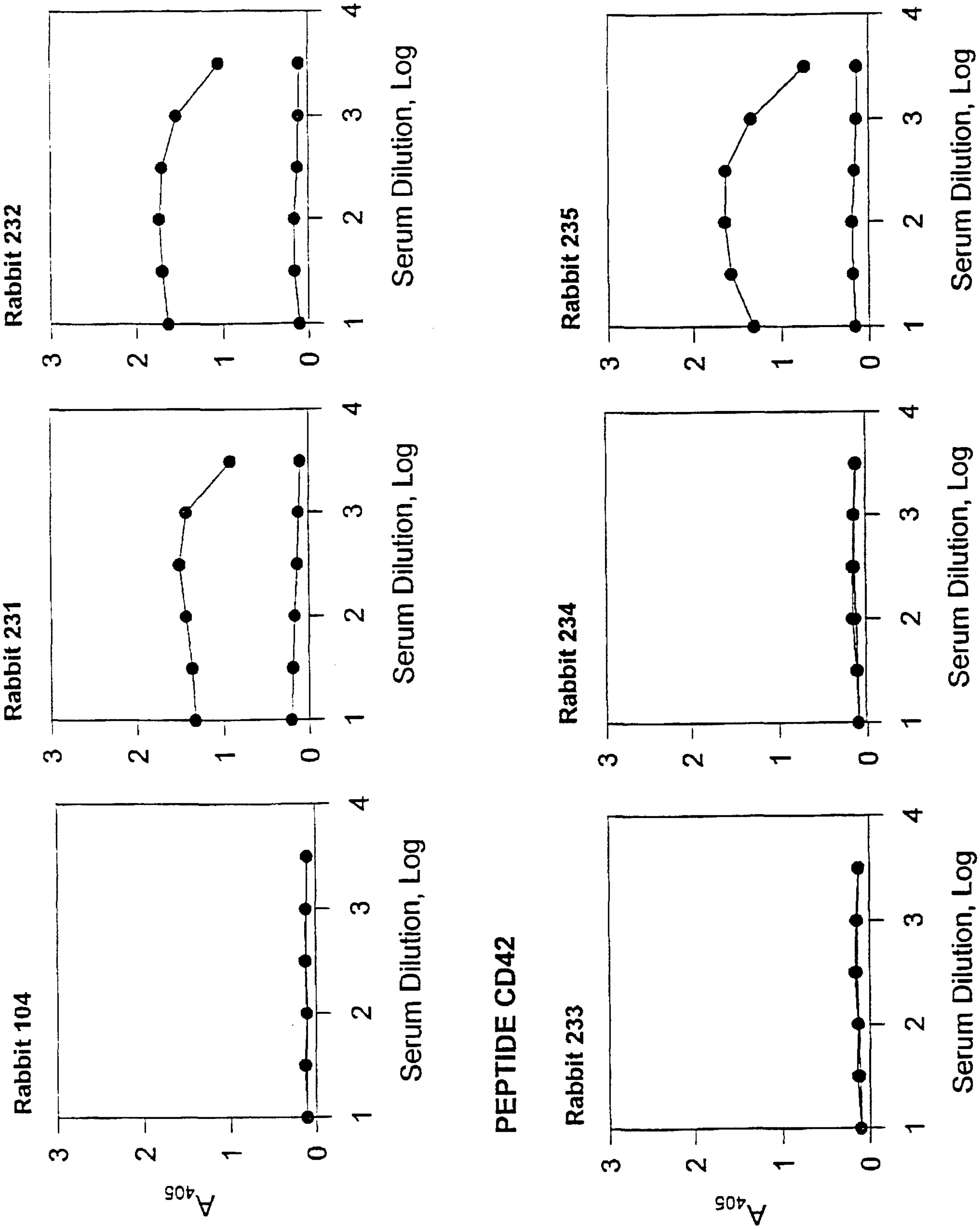


FIGURE 10D

Data courtesy of Ms. Mary Kay Gentry WRAIR

FIGURE 10E



Data courtesy of Ms. Mary Kay Gentry WRIAR

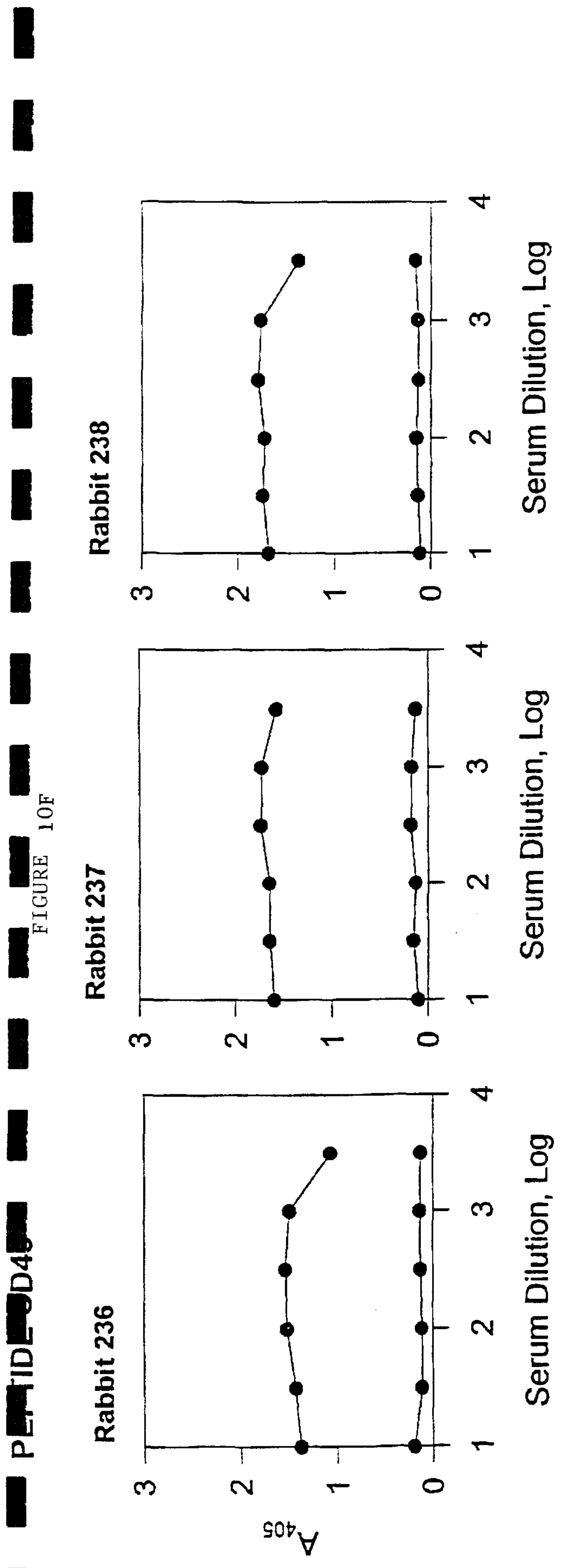
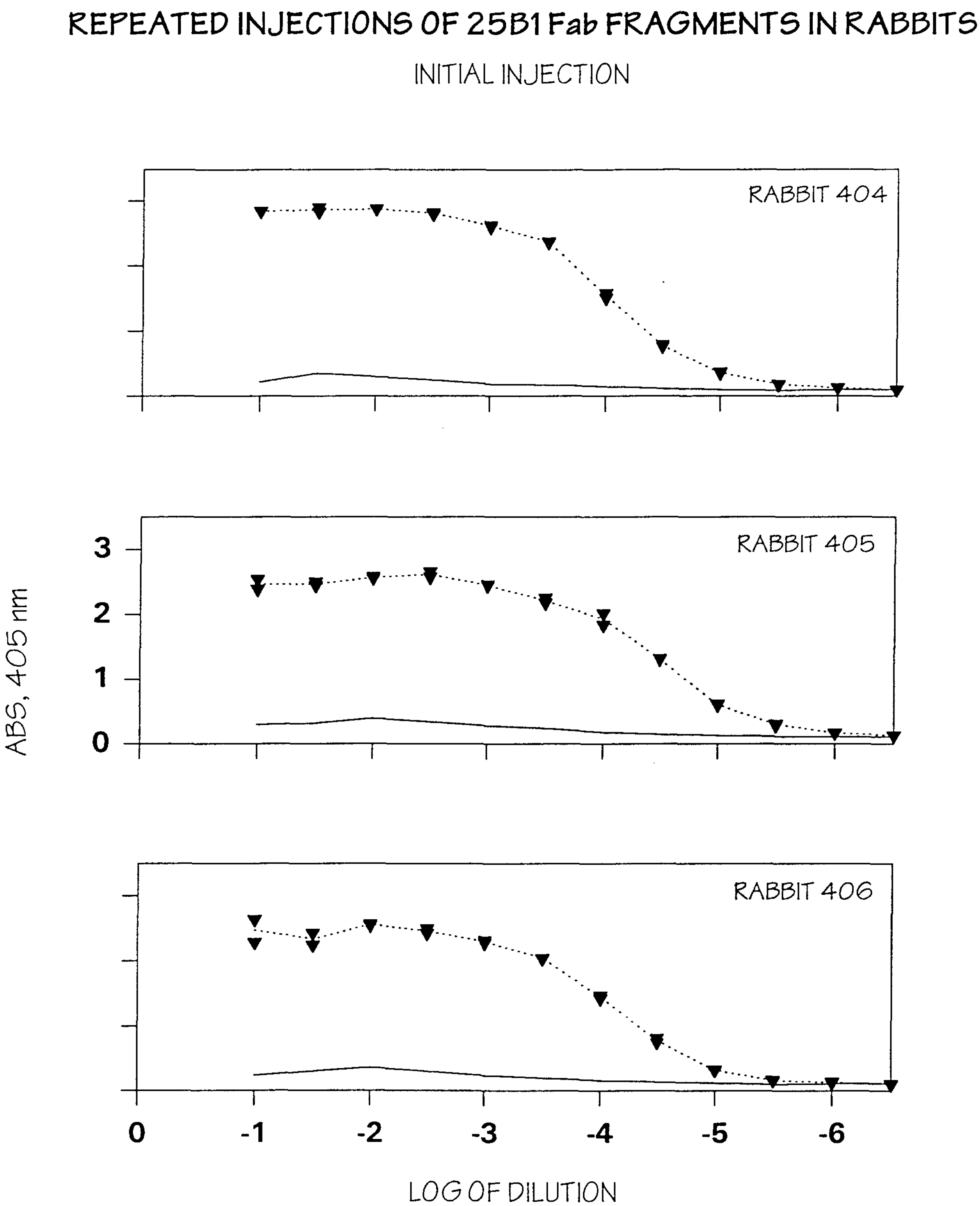


FIGURE 10F

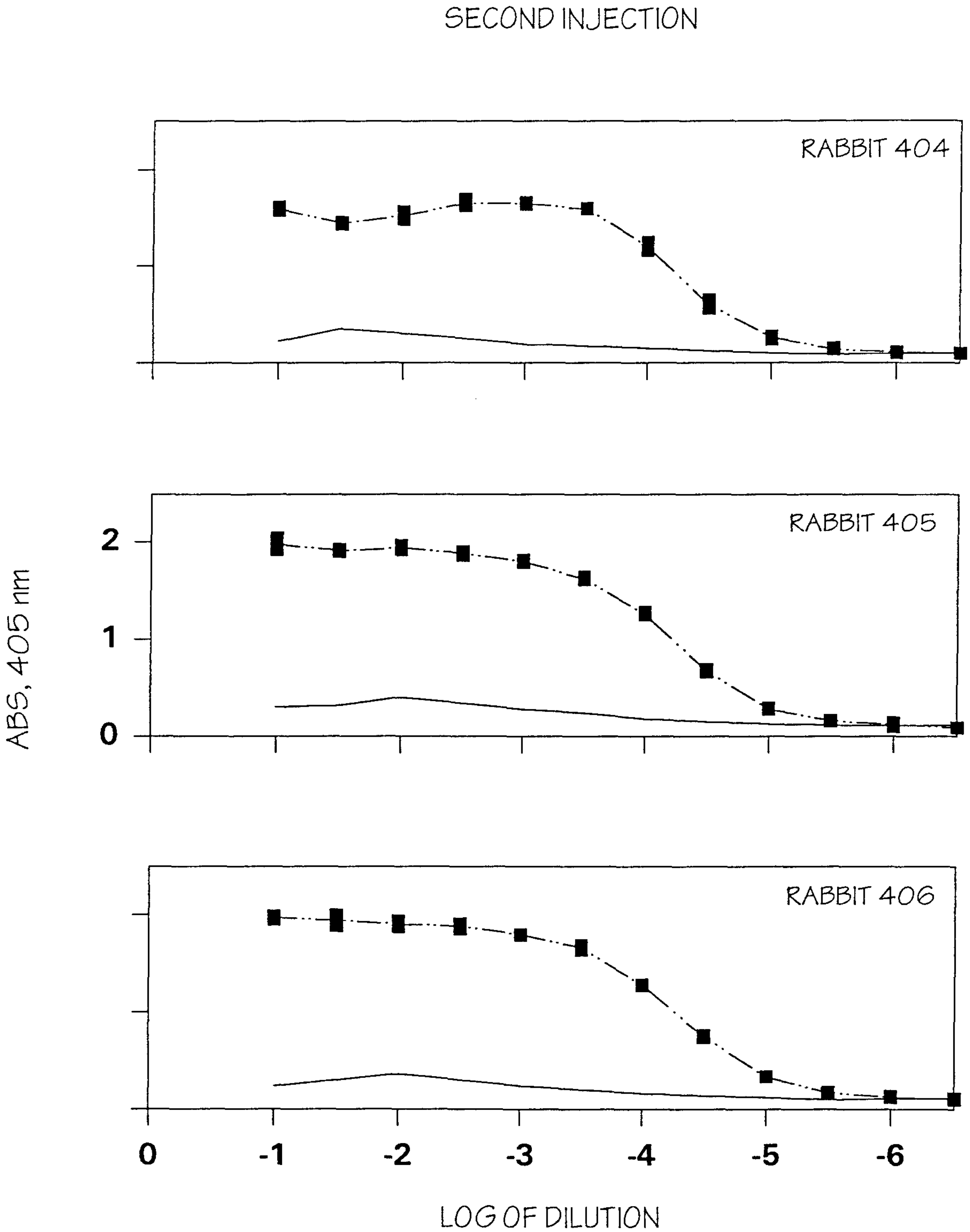
Data courtesy of Ms. Mary Kay Gentry WRAIR

FIGURE 11A



Data courtesy of Ms. Mary Kay Gentry WRAIR

REPEATED INJECTIONS OF 25B1 Fab FRAGMENTS IN RABBITS

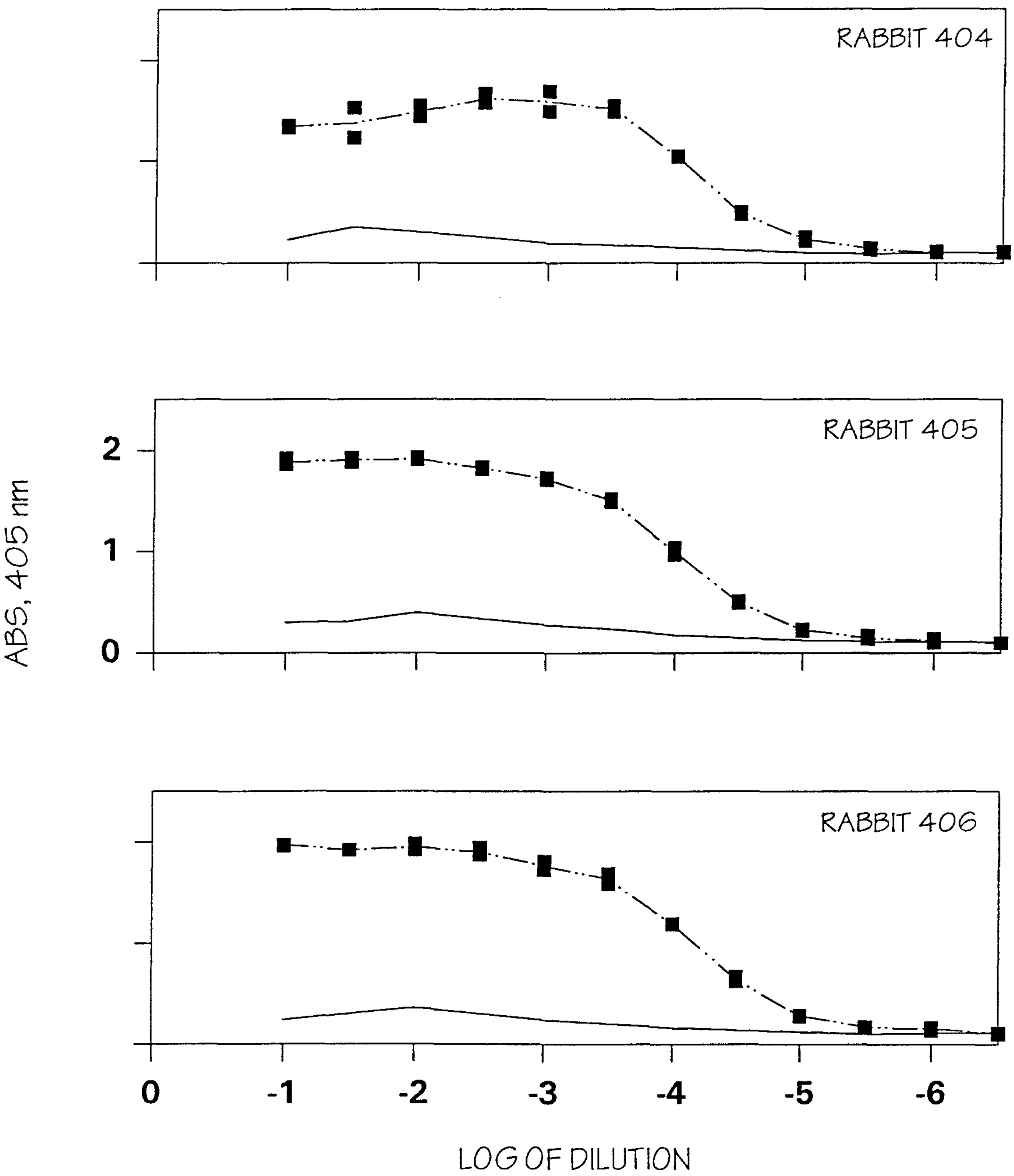


Data courtesy of Ms. Mary Kay Gentry WRAIR

FIGURE 11C

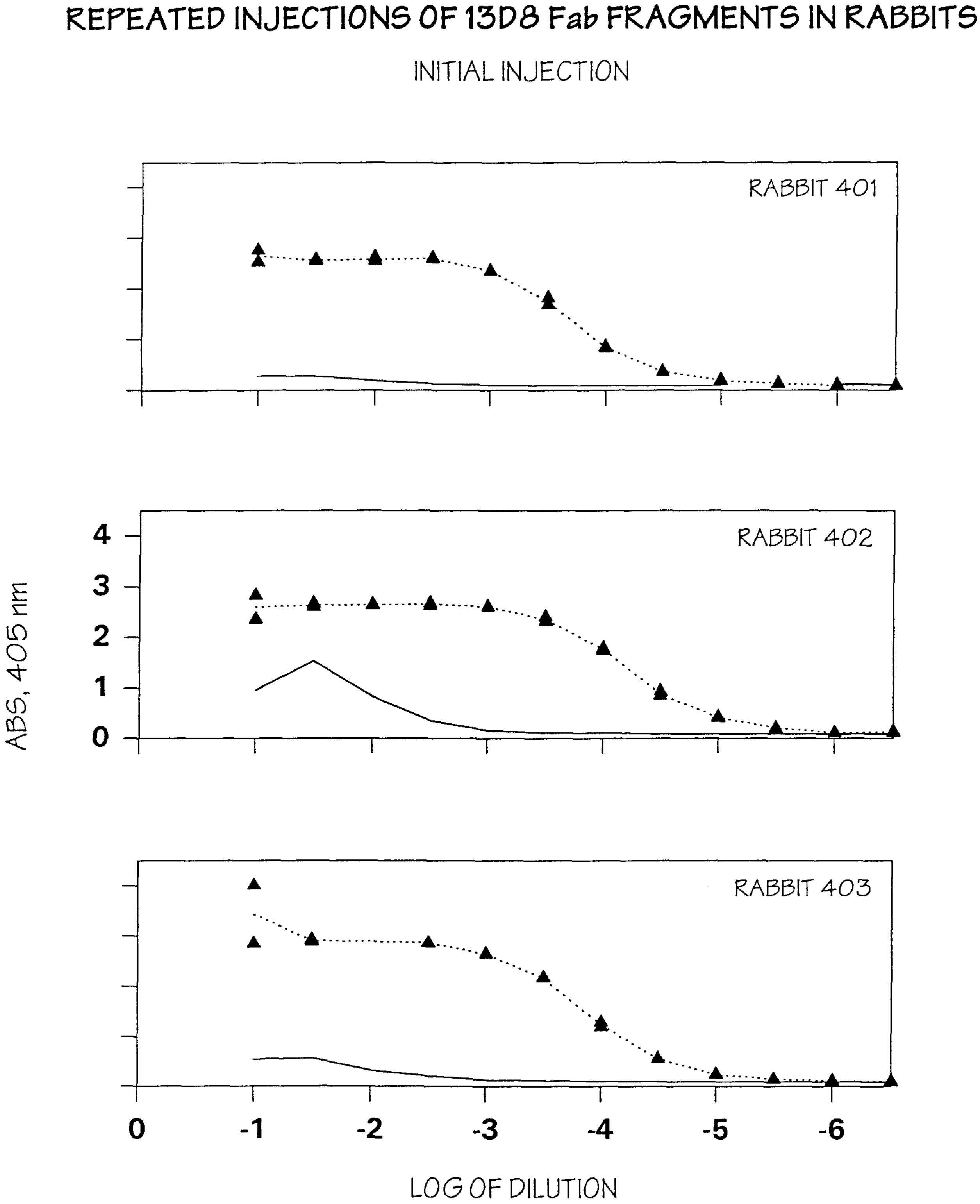
REPEATED INJECTIONS OF 25B1 Fab FRAGMENTS IN RABBITS

FINAL INJECTION



Data courtesy of Ms. Mary Kay Gentry WRAIR

FIGURE 11D

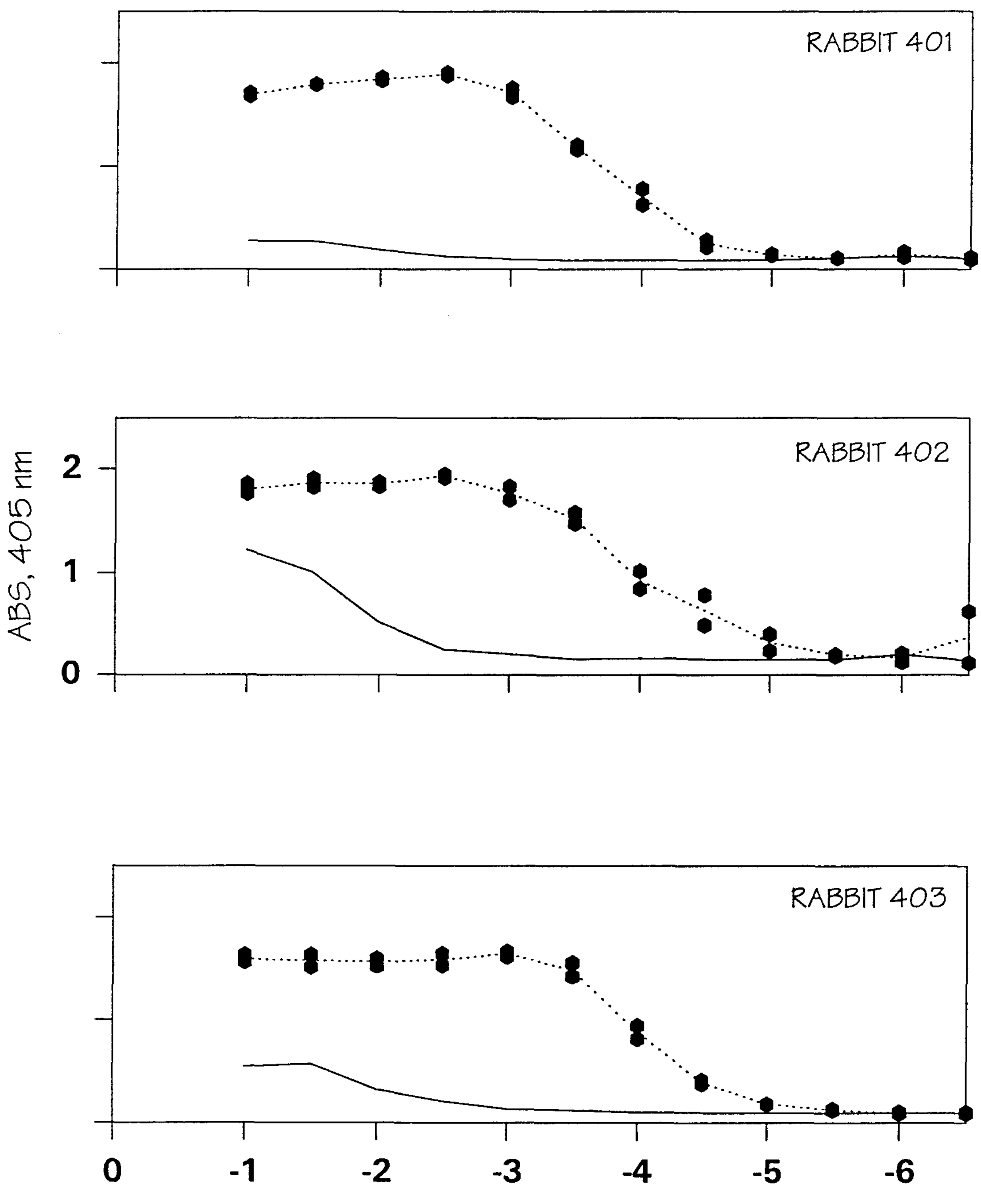


Data courtesy of Ms. Mary Kay Gentry WRAIR

FIGURE 11E

REPEATED INJECTIONS OF 13D8 Fab FRAGMENTS IN RABBITS

FINAL BLEED



Data courtesy of Ms. Mary Kay Gentry WRAIR

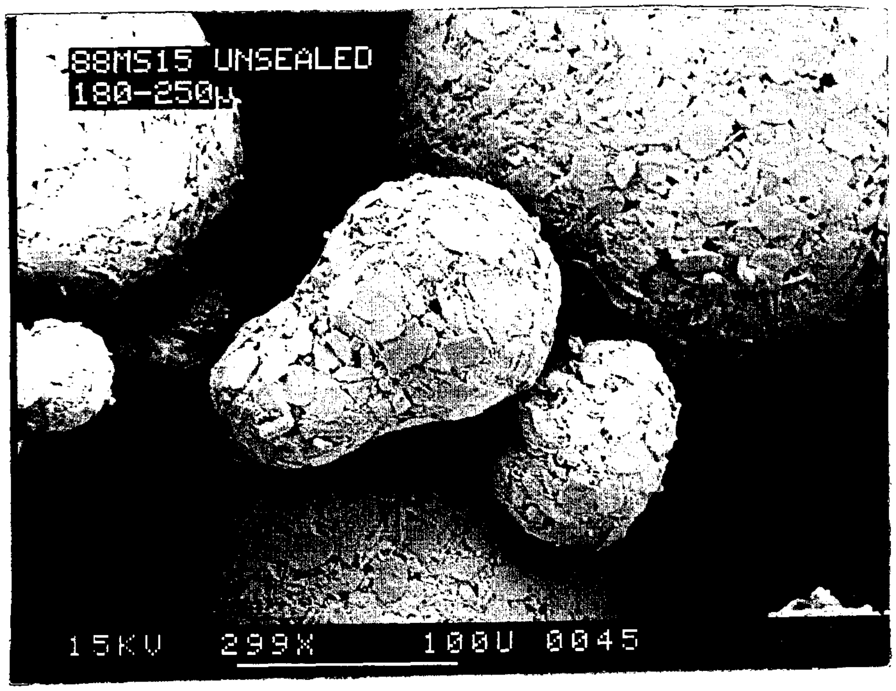


FIGURE 12A - Scanning electron micrograph of BChE microsphere Batch 88MS15 (299X).

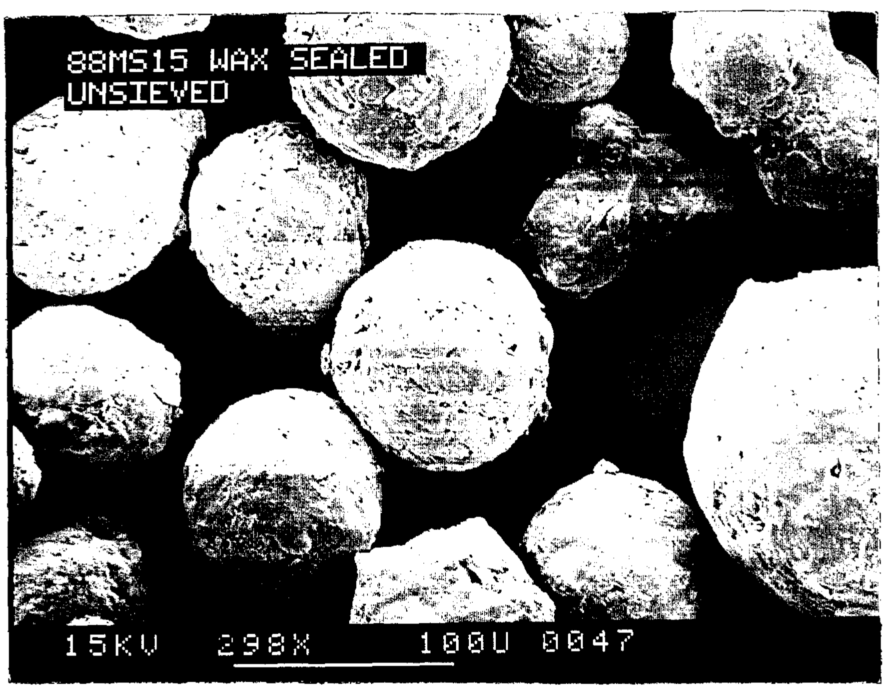


FIGURE 12B - Scanning electron micrograph of BChE microsphere Batch 88MS15 wax sealed (298X).

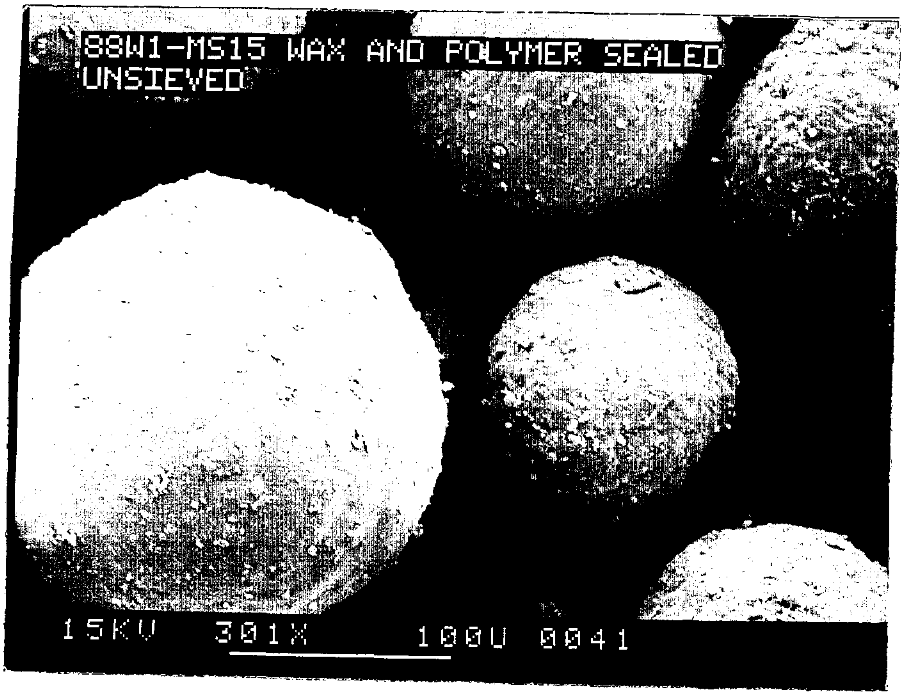


FIGURE 12C - Scanning electron micrograph of BChE microsphere Batch 88W1-MS15 wax coated and PLGA coated (301X).

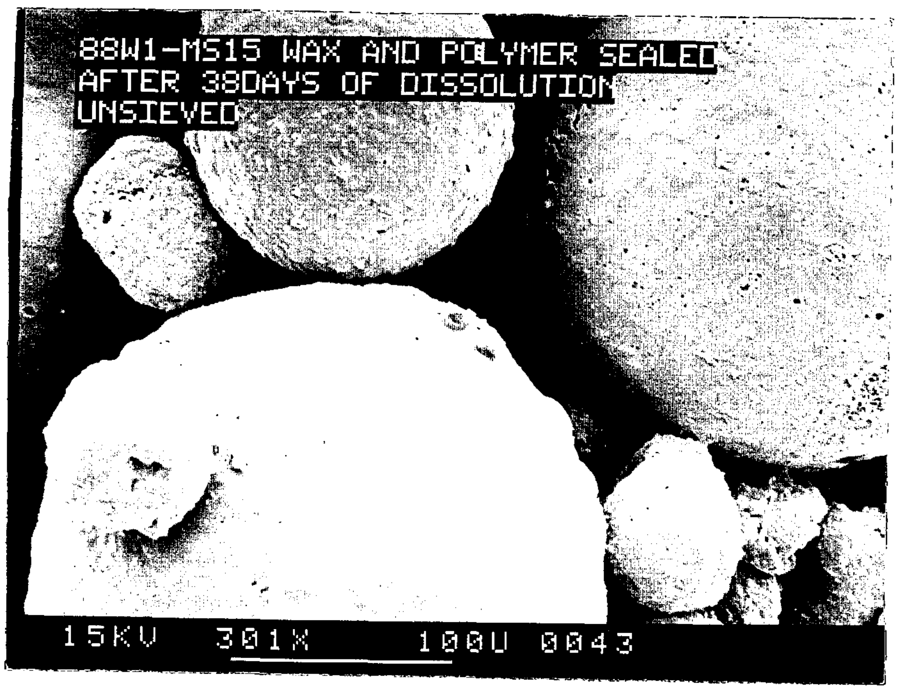


FIGURE 12D - Scanning electron micrograph of BChE microsphere Batch 88W1 after 38 days in distilled water (301X).

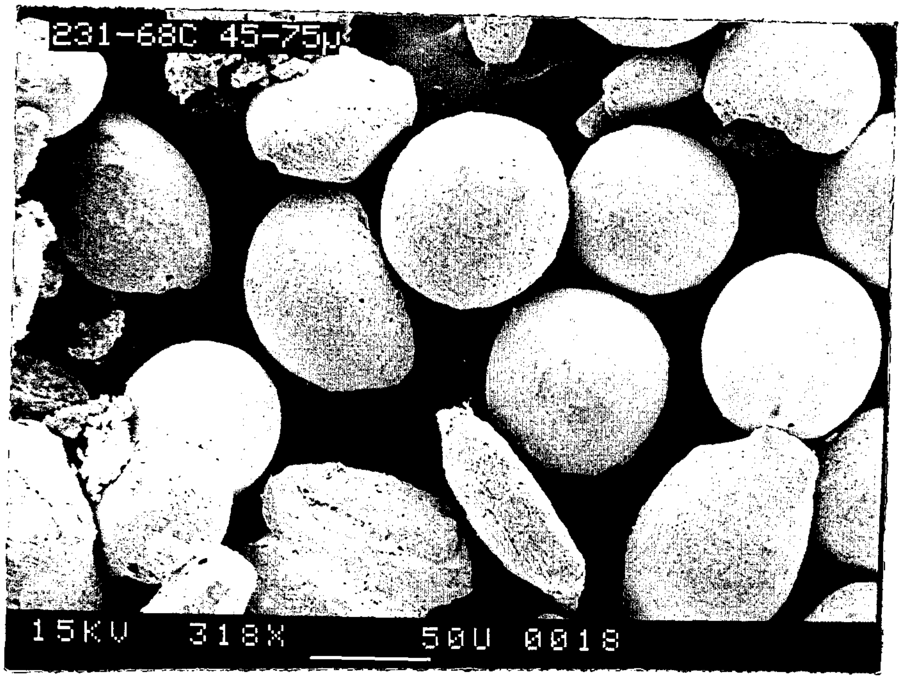
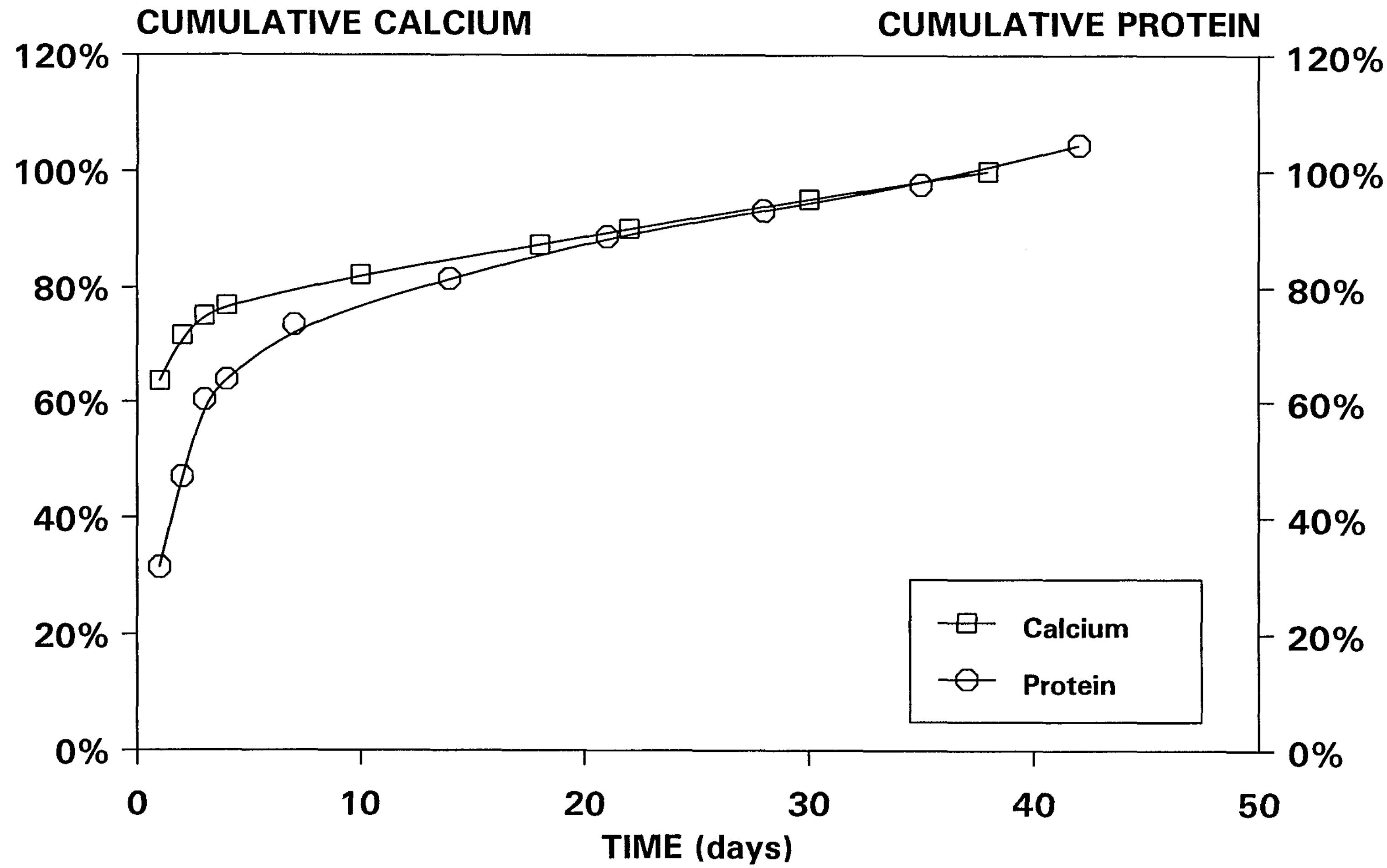


FIGURE 13 Scanning electron micrograph of mineral microspheres containing BChE (Batch 72MSG5 - 45-75 μm). Magnification 318X.

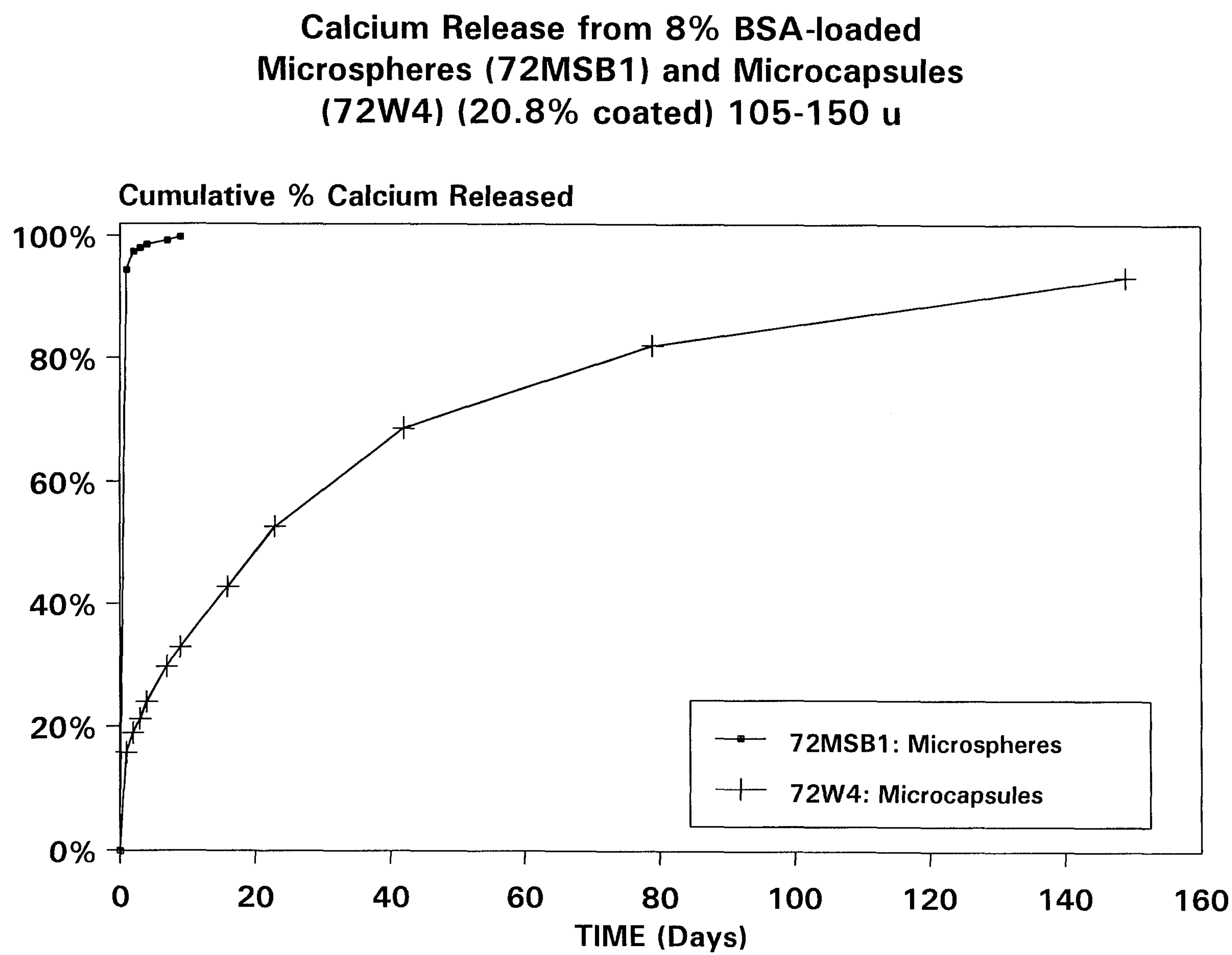
FIGURE 14

Release of Calcium and Protein from Hydroformed Calcium Sulfate Microcapsule Formulation 88W1 (53-250um)



247#2188-R4

FIGURE 15



#39423196%

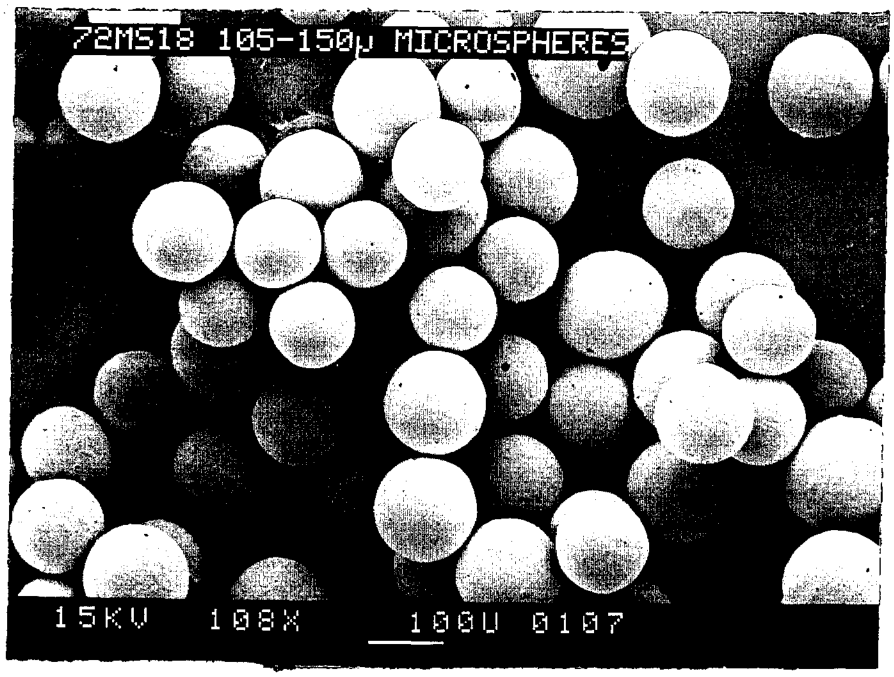
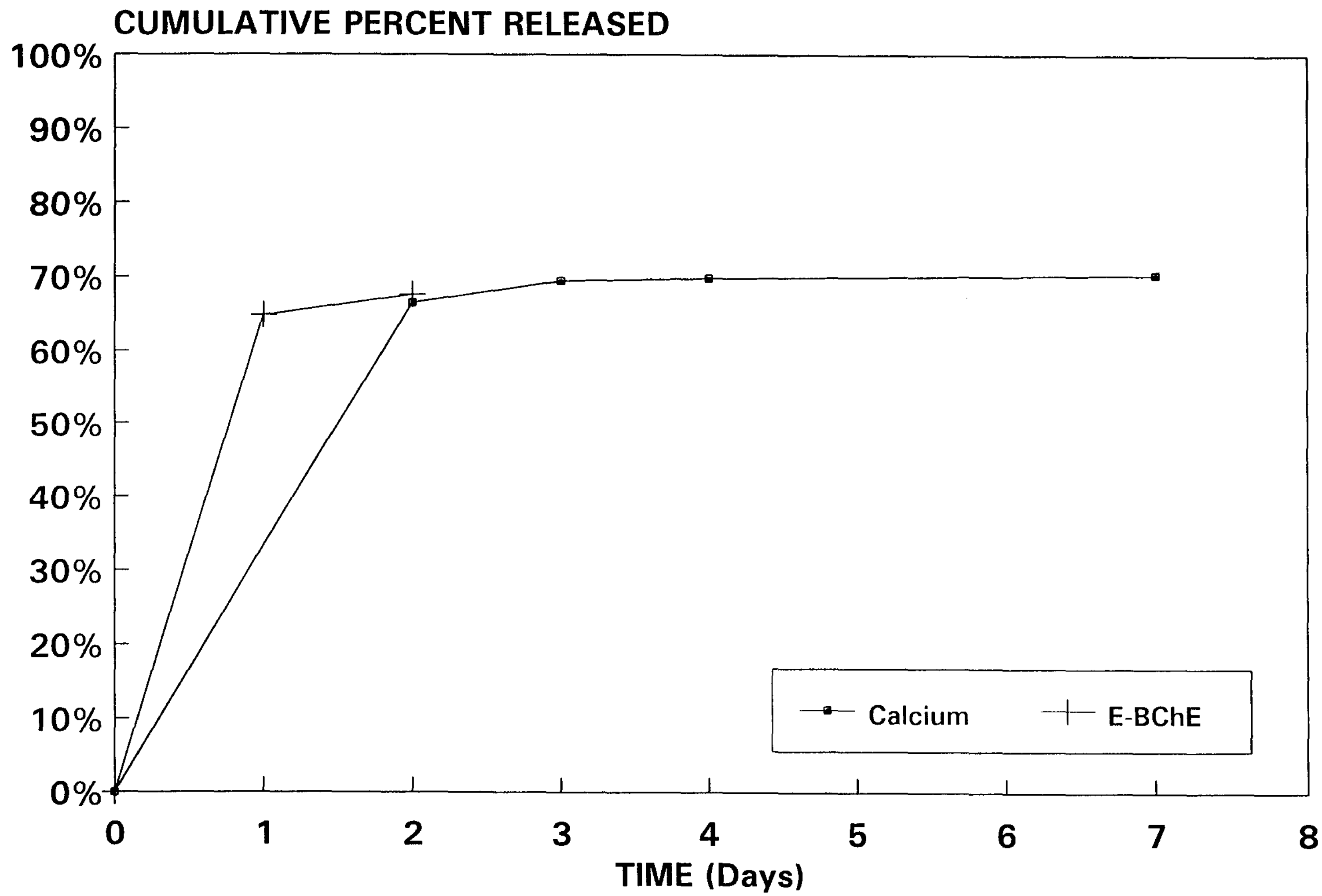


FIGURE 16 - Scanning electron micrograph of Batch 72MS18 E-BChE microcapsules prepared by the water-in-oil-in-water method (108X).

FIGURE 17

Release of Calcium and E-BChE from W/O/W Microcapsules (72MS18)



436:finrpt1

FIGURE 18A

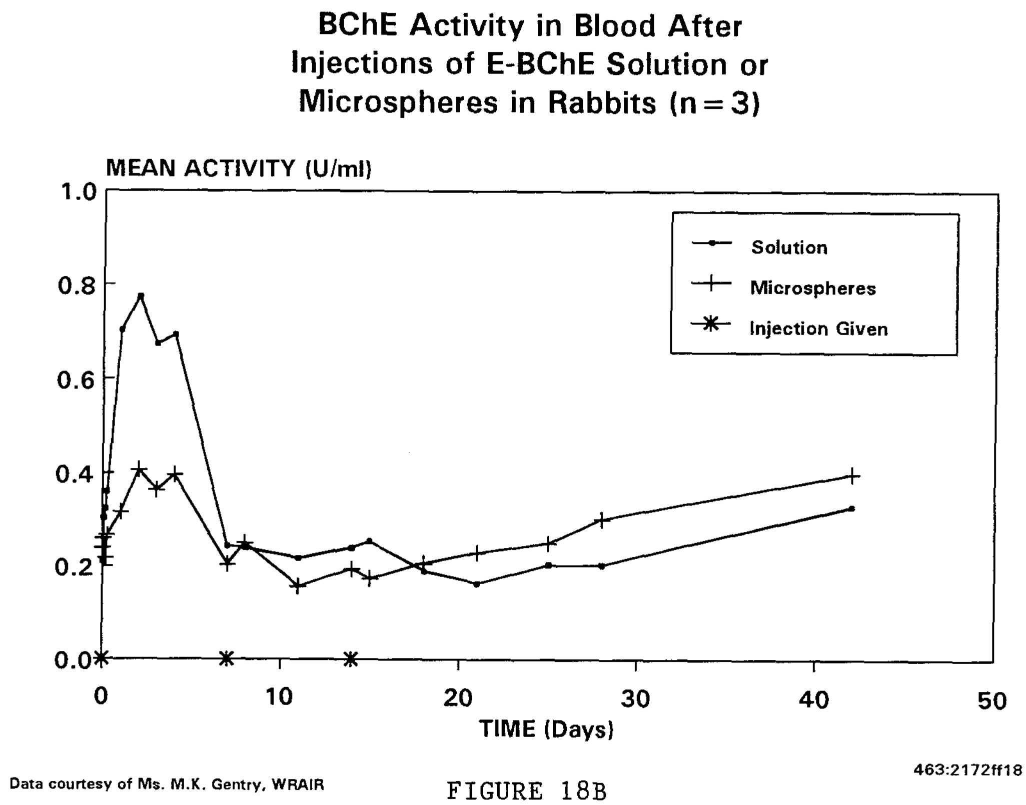


FIGURE 18B

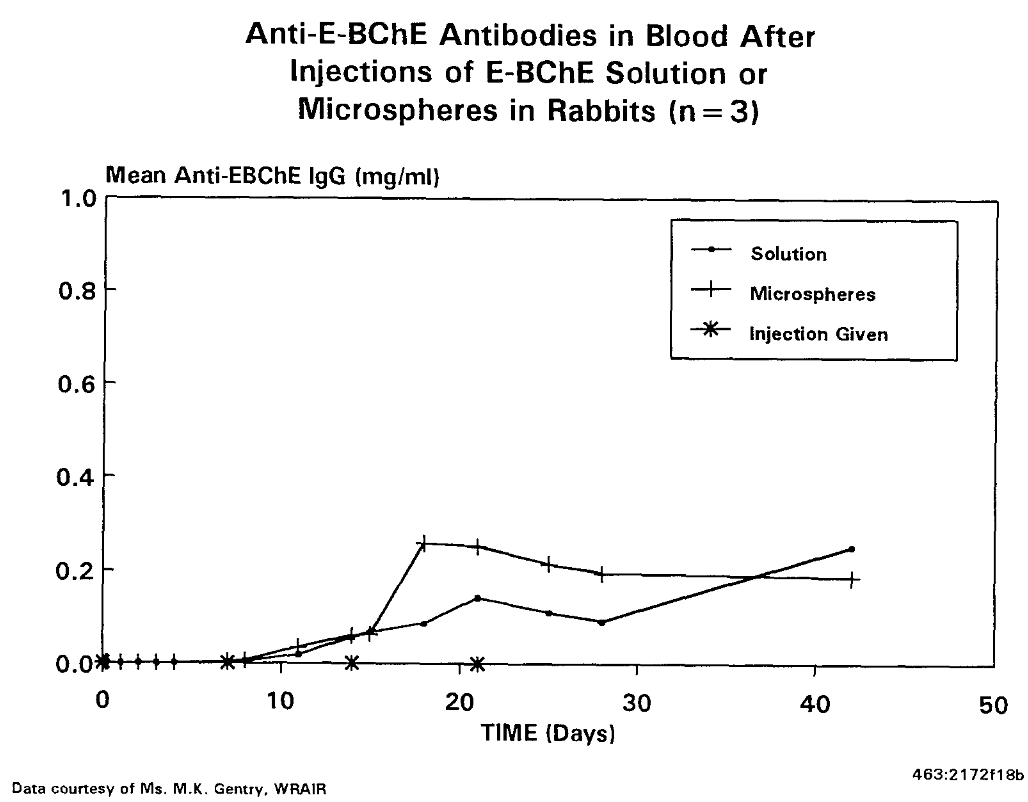
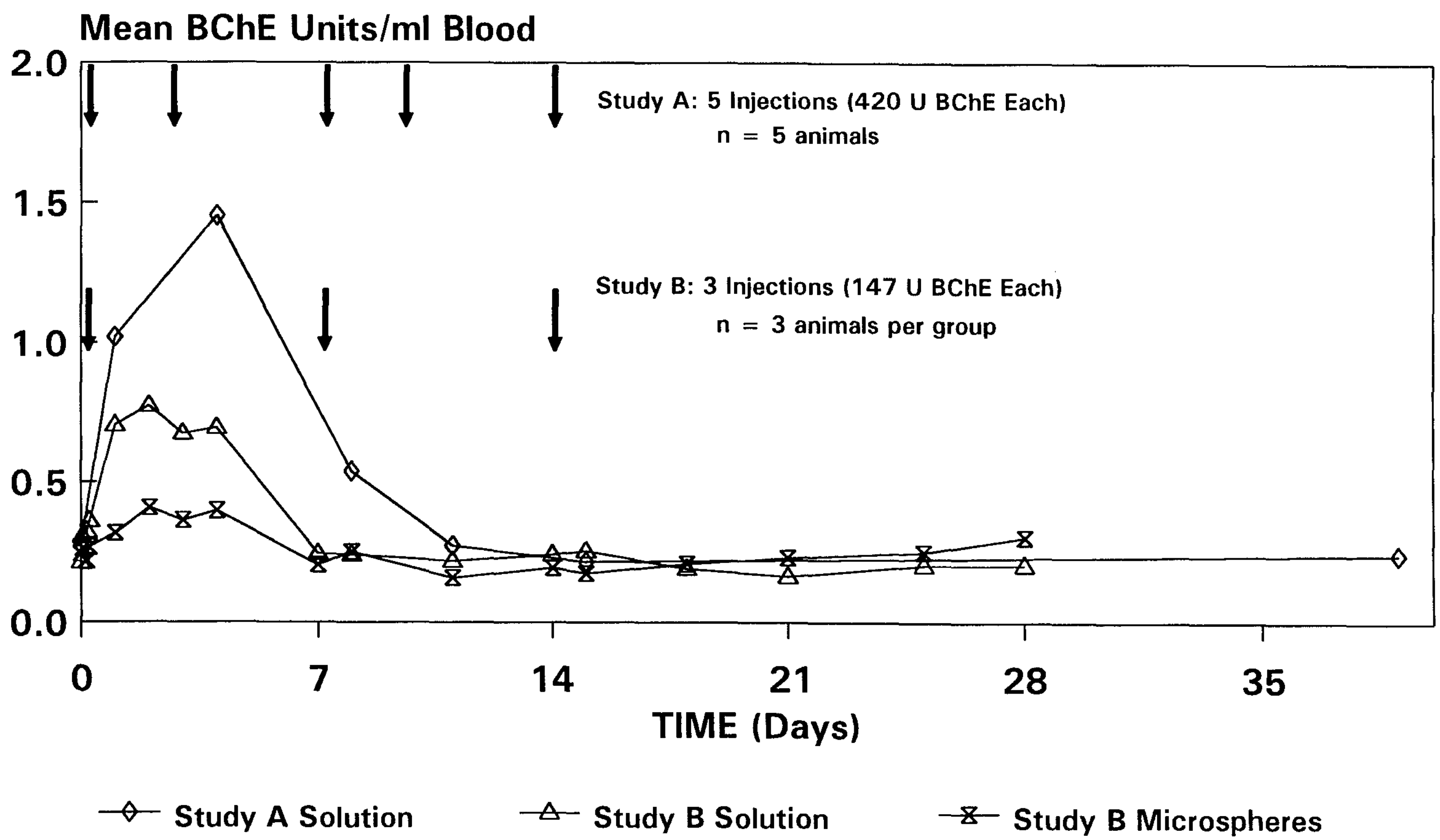


FIGURE 19

BChE Blood Levels in Rabbits After Repeated Injections of Microspheres or Solutions



Data courtesy of Ms. M.K. Gentry, WRAIR

394:nbche3

APPENDIX A

AUTOPSY AND HISTOPATHOLOGY REPORTS

IT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

EQUEST FOR:
) Complete Necropsy
) Gross Necropsy Only
) Biopsy

Accession # 95-1414
Charge Account# Biotek
Date 5/17/95

NVESTIGATOR	ANIMAL	ENVIRONMENT
me E.S. Nuwayser	ID# #96	Supplier
nst Biotek Dept	Species Rabbit	Date Rec'd
ddress 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Biotek Rm
Woburn, MA 01801	( )M (X)F Age	Date of Necropsy 5/18/95
hone	( ) Neutered	

ummary of Experimental Manipulations: Injected previously with BCHE I.M. and S.Q.: on 5/15/95 BCHE njected I.V.

Clinical History:
X)Died ( )Euthanized - Method: Date of Death: 5/16/95

Found dead.

Clinician: Fox

ifferential Diagnosis:

ross Necropsy Findings:

1. Minimal congestion of subcutaneous vasculature.
2. Blood in fur surrounding external nares.
3. Heart distended with blood and flabby.
4. Both kidneys are soft.
5. Postmortem discoloration of the gastrointestinal tract and uterine horns.
6. All other organ systems appear to be within normal limits.

Prosector: Pang

Histopathologic Observations:

.V.L.:

Cause of Death:

ode:

Comment:

James C. Murphy, D.V.M., Ph.D.
Veterinary Pathologist

(5/95) db
LABORATORY REQUESTS:
)Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
)Para ( )Urin ( )Clin Chem ( )Micro

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
( ) Gross Necropsy Only
( ) Biopsy

Accession # 95-1415
Charge Account# Biotek
Date 5/18/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name E.S. Nuwayser	ID# #97	Supplier
Inst Biotek Dept	Species Rabbit	Date Rec'd
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Biotek Rm
Woburn, MA 01801	( )M (X)F Age	Date of Necropsy 5/17/95
Phone	( ) Neutered	

Summary of Experimental Manipulations: Injected previously with BCHE I.M. and S.C.; on 5/15/95 BCHE injected I.V.

Clinical History:
(X)Died ( )Euthanized - Method: Date of Death: 5/16/95

Found dead 5/16/95.

Clinician: Fox

Differential Diagnosis:

Gross Necropsy Findings:

1. Blood in the fur surrounding the external nares.
2. Diffuse congestion of all subcutaneous vasculature with focal areas suggestive of hemorrhage associated with many vessels.
3. Both retropharyngeal lymph nodes are red.
4. All lobes of lung are mottled shades of red.
5. A few 1 mm diameter yellow foci are on the surface of the left lobe of liver at the perimeter.
6. Postmortem color changes on the ovaries, uterus and gastrointestinal tract.
7. All other organ systems appear to be within normal limits.

Prosector: Murphy

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

James C. Murphy, D.V.M., Ph.D.
Veterinary Pathologist
JCM

(5/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1416

Charge Account# Biotek
Date 5/18/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name E.S. Nuwayser	ID# #98	Supplier
Inst Biotek Inc.	Species Rabbit	Date Rec'd
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Biotek Rm
Woburn, MA 01801	( )M (X)F Age	Date of Necropsy 5/17/95
Phone	( ) Neutered	

Summary of Experimental Manipulations: Injected previously with BCHE I.M. and S.Q.; on 5/15/95 BCHE injected I.V.

Clinical History:
(X)Died ( )Euthanized - Method: Date of Death: 5/16/95
Found dead 5/16/95.

Clinician: Fox

Differential Diagnosis:

Gross Necropsy Findings:

1. Blood in the fur surrounding the external nares.
2. Congestion of vessels on the ventral abdomen in the subcutis and a small accumulation of fluid in the same area.
3. Retropharyngeal lymph nodes and mesenteric lymph nodes are dark red.
4. Accumulation of dark serosanguinous fluid within the pericardial sac.
5. All chambers of heart are distended with blood.
6. The liver is brown with yellow mottling on cut surfaces. The lungs are mottled shades of red.
7. Postmortem color changes throughout the gastrointestinal tract and uterus. Both kidneys soft.
8. All other organ systems appear to be within normal limits.

Prosector: Murphy

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

James C. Murphy, D.V.M., Ph.D.
Veterinary Pathologist JCM

(5/95) jb
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1455
Charge Account# Biotek
Date 5/24/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name E.S. Nuwayser	ID# 99	Supplier HRP
Inst Biotek Dept	Species Rabbit	Date Rec'd 5/11/95
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Rm
Woburn, MA 01801	( )M (X)F Age Adult	Date of Necropsy 5/24/95
Phone 617 938-0938	( ) Neutered	

Summary of Experimental Manipulations: Injected with BchE and AchE intramuscularly and AchE intraveneously.

Clinical History:
( )Died (X)Euthanized - Method: I.V. Somlethol (1cc) Date of Death: 5/24/95

SPF rabbit used in experimental study as above.

Clinician: Fox

Differential Diagnosis:

--

Gross Necropsy Findings: None.

Prosector:

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(5/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ________

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
( ) Gross Necropsy Only
( ) Biopsy

Accession # 95-1417
Charge Account# Biotek
Date 5/18/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name E.S. Nuwayser	ID# #100	Supplier
Inst Biotek Inc.	Species Rabbit	Date Rec'd
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Biotek Rm
Woburn, MA 01801	( )M (X)F Age	Date of Necropsy 5/17/95
Phone	( ) Neutered	

Summary of Experimental Manipulations: Injected previously with BCHE I.M. and S.Q.: on 5/15/95 BCHE injected I.V.

Clinical History:
( )Died (X)Euthanized - Method: Somlethal Date of Death: 5/16/95
Euthanized 5/17/95. Labored breathing, cyanosis evidenced by bluish mucus membranes and ears. Temp 101°: depressed. Responded to pinch on foot.

Clinician: Fox

Differential Diagnosis: Presumptive anaphylopia.

Gross Necropsy Findings:

1. Diffuse congestion of all subcutaneous vasculature with focal areas suggestive of hemorrhage associated with numerous vessels.
2. Some postmortem color changes of ovaries, uterus and gastrointestinal tract.
3. All other organ systems appear to be within normal limits.

Prosector: Pagn

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

James C. Murphy, D.V.M., Ph.D.
Veterinary Pathologist JCM

(5/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1456
Charge Account# Biotek
Date 5/24/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name E.S. Nuwayser	ID# 443	Supplier HRP
Inst Biotek Dept	Species Rabbit	Date Rec'd 1/31/95
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Rm
Woburn, MA 01801	( )M (X)F Age Adult	Date of Necropsy 5/24/95
Phone 617 938-0938	( ) Neutered	

Summary of Experimental Manipulations: Intravenously injected with AchE only one time.

Clinical History:
( )Died (X)Euthanized - Method: I.V. Somlethol (1cc) Date of Death: 5/24/95

SPF rabbit for experimental study.

Clinician: Fox

Differential Diagnosis:

Gross Necropsy Findings: None.

Prosector:

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(5/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ______

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1753
Charge Account# Biotek
Date 7/17/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name Lee Nuwayser	ID# 105	Supplier HRP
Inst Biotek Dept	Species Rabbit	Date Rec'd 5/11/93
Address 21 C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Rm
Woburn, MA 01801	( )M (X)F Age 2.5 years	Date of Necropsy 7/17/95
Phone 938-0938	( ) Neutered	

Summary of Experimental Manipulations: AchE Study, ½ feed. Animal was injected with AchE 2 years ago intramuscularly.

Clinical History:
( )Died (X)Euthanized - Method: Somlethol Date of Death: 7/17/95

Tissues to be returned to investigators:
1. Kidney
2. Liver
3. Spleen
4. Heart
5. Urinary bladder
6. Lymph nodes

Clinician: Nuwayser/Musto/Fox

Differential Diagnosis:

Gross Necropsy Findings:

No significant lesions.

Prosector: Pang

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(7/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ______

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1754

Charge Account# Biotek
Date 7/17/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name Lee Nuwavser	ID# 106	Supplier HRP
Inst Biotek Dept	Species Rabbit	Date Rec'd 5/11/93
Address 21 C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Rm
Woburn. MA 01801	( )M (X)F Age 2.5 years	Date of Necropsy 7/17/95
Phone 938-0938	( ) Neutered	

Summary of Experimental Manipulations: AchE Study, ½ feed. Animal was injected intramuscularly with AchE two years ago.

Clinical History:
( )Died (X)Euthanized - Method: Somlethol Date of Death: 7/17/95

Tissues to be returned to investigators:
1. Kidney
2. Liver
3. Spleen
4. Heart
5. Urinary bladder
6. Lymph nodes

Clinician: Nuwayser/Musto/Fox

Differential Diagnosis:

Gross Necropsy Findings:

No significant lesions.

Prosector: Pang

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(7/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ____________

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1755
Charge Account# Biotek
Date 7/17/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name Lee Nuwayser	ID# 107	Supplier HRP
Inst Biotek Dept	Species Rabbit	Date Rec'd 5/11/93
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg ____ Rm ____
Woburn, MA 01801	( )M (X)F Age 2.5 years	Date of Necropsy 7/17/95
Phone 938-0938	( ) Neutered	

Summary of Experimental Manipulations: AchE Study, ½ feed. Animal was injected intramuscularly with AchE two years ago.

Clinical History:
( )Died (X)Euthanized - Method: Somlethol Date of Death: 7/17/95

Tissues to be returned to investigators:

1. Kidney
2. Liver
3. Spleen
4. Heart
5. Urinary bladder
6. Lymph nodes

Clinician: Nuwayser/Musto/Fox

Differential Diagnosis:

Gross Necropsy Findings:

A 2 x 3 x 1.5 cm meaty mass on the endometrium of the left uterine horn.

Prosector: Pang

Histopathologic Observations:

N.V.L.:

Diagnosis: Uterine tumor, presumptively.

Code:

Comment:

XL

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(7/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ________

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1756
Charge Account# Biotek
Date 7/17/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name Lee Nuwayser	ID# 108	Supplier HRP
Inst Biotek Dept	Species Rabbit	Date Rec'd 5/11/93
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg ___ Rm ___
Woburn, MA 01801	( )M (X)F Age 2.5 years	Date of Necropsy 7/17/95
Phone 938-0938	( ) Neutered	

Summary of Experimental Manipulations: AchE Study, ½ feed. Animal was injected intramuscularly with AchE two years ago.

Clinical History:
( )Died (X)Euthanized - Method: Somlethol Date of Death: 7/17/95

Tissues to be returned to investigators:
1. Kidney
2. Liver
3. Spleen
4. Heart
5. Urinary bladder
6. Lymph nodes

Clinician: Nuwayser/Musto/Fox

Differential Diagnosis:

Gross Necropsy Findings:

No significant lesions.

Prosector: Pang

Histopathologic Observations:

N.V.L.:

Diagnosis:

Code:

Comment:

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(7/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ____________

MIT - DCM

DIL

REQUEST FOR PATHOLOGY SERVICE

REQUEST FOR:
( ) Complete Necropsy
(X) Gross Necropsy Only
( ) Biopsy

Accession # 95-1757
Charge Account# Biotek
Date 7/17/95

INVESTIGATOR	ANIMAL	ENVIRONMENT
Name Biotek/ESN	ID# 109	Supplier HRP
Inst Dept	Species Rabbit	Date Rec'd 5/11/93
Address 21-C Olympia Avenue	Breed/Strain NZW	Housed in Bldg Rm
Woburn, MA 01801	( )M (X)F Age 2.5 years	Date of Necropsy 7/17/95
Phone 938-0938	( ) Neutered	

Summary of Experimental Manipulations: Ache Study, ½ feed. Animal was injected intramuscularly with AchE two years ago.

Clinical History:
( )Died (X)Euthanized - Method: Somlethol Date of Death: 7/17/95

Tissues to be returned to investigators:
1. Kidney
2. Liver
3. Spleen
4. Heart
5. Urinary bladder
6. Lymph nodes

Clinician: Nuwayser/Musto/Fox

Differential Diagnosis:

Gross Necropsy Findings:

No significant lesions.

Prosector: Pang

Histopathologic Observations:

N.V.L.:

Cause of Death:

Code:

Comment:

Xiantang Li, D.V.M., Ph.D.
Veterinary Pathologist

(7/95) db
LABORATORY REQUESTS:
( )Micro ( )Hema ( )Serology ( )Specimen Frozen Photos: ( )Gross
( )Para ( )Urin ( )Clin Chem ( )Micro
( ) ________

RECEIVED
JUL 31 1995

DOCUMENT TRANSMITTAL

FROM: ROBERT F. McCONNELL, D.V.M., P.A.

ADDRESS: 12 CHERRYVILLE RD., FLEMINGTON, NJ 08822

RFMc STUDY NO.: 146/95

BIOTEK STUDY NO.: 2172

DESCRIPTION: MICROSCOPIC EVALUATION OF SELECTED TISSUES FROM RABBITS WHICH DIED OR WERE KILLED *IN EXTREMIS*. Histopathology report and slides (42).

SIGNED: Sharon McConnell DATE: 7/25/95

ROBERT F. McCONNELL, D.V.M., P.A.
CONSULTING PATHOLOGY SERVICES
PHONE 908-782-4676

DIPLOMATE
AMERICAN COLLEGE
OF
VETERINARY PATHOLOGISTS

12 CHERRYVILLE ROAD
FLEMINGTON, NEW JERSEY 08822

July 24, 1995

RECEIVED
JUL 31 1995

TO: E. S. NUWAYSER, Ph.D.
BIOTEK, INC.
21-C OLYMPIA AVE.
WOBURN, MA 01801

RE: BIOTEK STUDY #2172. MICROSCOPIC EVALUATION OF SELECTED TISSUES FROM RABBITS WHICH DIED OR WERE KILLED *IN EXTREMIS.*

Selected hematoxylin and eosin stained tissues from 6 rabbits, which died or were killed moribund, of the aforementioned study were submitted for microscopic examination. The tissues from each rabbit included heart, liver, spleen, kidney, urinary bladder, retropharyngeal and mesenteric lymph nodes.

Table 1 lists the rabbits and the microscopic findings.

Microscopic examination of the tissues failed to define a probable cause of death or morbidity.

The mesenteric and retropharyngeal lymph nodes of rabbits #96, 97, 98, and 100 had varying degrees of lymphoid depletion while lymphoid follicular hypertrophy/hyperplasia was present in rabbits #99 and 443. Increased hemosiderin pigment within splenic macrophages was noted in 5/6 rabbits and was especially prominent in rabbit #99.

Lymphocytic cholangiolitis was present in the liver of rabbit #99. This change was judged to have been related to nosematosis, a protozoan parasitic infection in rabbits.

In conclusion, the microscopic evaluation of selected tissues from 6 rabbits of this study failed to define the probable cause of death or morbidity. There was, however, no evidence of an infectious agent as having been the cause of the mortalities.

Respectfully submitted,

Robert F. McConnell

ROBERT F. McCONNELL, D.V.M.

146/95

BIOTEK, INC.
STUDY #2172

MICROSCOPIC FINDINGS

TABLE 1

ANIMAL NO.	96	97	98	99	100	443
TISSUE/RESPONSE						
HEART	N	N		N	N	N
Postmortem bacterial growth	-	-	P	-	-	-
LIVER		N				N
Postmortem bacterial growth	P	-	P	-	-	-
Lymphocytic cholangiolitis	-	-	-	<2>	-	-
Venous congestion	-	-	-	-	P	-
SPLEEN						
Autolysis	3	3	3	-	-	-
Lymphoid depletion	1	2	2	-	2	-
Increased hemosiderin pigment (phagocytized)	1	-	1	3	2	1
Lymphoid follicular hypertrophy/hyperplasia	-	-	-	2	-	3
KIDNEY				N	N	N
Postmortem bacterial growth	P	P	-	-	-	-
Autolysis	2	2	3	-	-	-
URINARY BLADDER	N	N	N	N	N	N
Autolysis	2	2	-	-	2	-
RETROPHARYNGEAL LYMPH NODE				N		
Lymphoid depletion	2	2	2	-	2	-
Hemorrhage	-	3	-	-	-	-
Autolysis	-	2	3	-	-	-
Lymphoid follicular hyperplasia	-	-	-	-	-	2
MESENTERIC LYMPH NODE	S	S		N		
Lymphoid depletion			2	-	2	-

96. 97 98, 100 - Injected previously with BCHE I.M. and S.Q.; on 5/15/95
BCHE - 96, 97, 98 - Died, 100 - Euthanized.
99 - Injected with BCHE and ACHE I.M. and ACHE I.V. - Killed *in extremis.*
443 - Injected I.V. with ACHE once - Killed *in extremis.*

LEGEND FOR TABLE
N = tissue within normal histological limits
1, 2. 3, 4 = degree of severity of indicated change
1 = minimal, 2 = mild, 3 = moderate, 4 = marked
< > = multifocal
S = insufficient tissue specimen for examination
P = indicated change present, grade not applicable
\- = indicated change not present

DOCUMENT TRANSMITTAL

FROM: ROBERT F. McCONNELL, D.V.M., P.A.

ADDRESS: 12 CHERRYVILLE RD., FLEMINGTON, NJ 08822

RFMc STUDY NO: 181/95

BIOTEK STUDY NO.: 2172A

DESCRIPTION: HISTOPATHOLOGICAL EVALUATION OF SELECTED TISSUES FROM RABBITS. Histopathology report and slides (23).

SIGNED: Ann McConnell DATE: 8/28/95

ROBERT F. McCONNELL, D.V.M., P.A.
CONSULTING PATHOLOGY SERVICES
PHONE 908-782-4676

DIPLOMATE
AMERICAN COLLEGE
OF
VETERINARY PATHOLOGISTS

12 CHERRYVILLE ROAD
FLEMINGTON, NEW JERSEY 08822

August 28, 1995

TO: E. S. NUWAYSER, Ph.D.
BIOTEK, INC.
21-C OLYMPIA AVE.
WOBURN, MA 01801

RE: BIOTEK STUDY #2172A. HISTOPATHOLOGICAL EVALUATION OF SELECTED TISSUES FROM RABBITS.

Selected hematoxylin and eosin stained tissues from 5 rabbits from Biotek Study 2172A were submitted for microscopic examination. The tissues included heart, spleen, liver, kidney, urinary bladder, and lymph nodes.

Table 1 lists the rabbits and the graded microscopic findings. Microscopic evaluation of the tissues revealed no evidence of a disease process or toxicologic activity. The spleen of the rabbits had varying depletion of lymphocytes and low level lymphoid follicular activity. Deposition of hemosiderin pigment was variable. No treatment associated trend, however, could be ascertained. The remaining tissues were within normal histological limits.

Respectfully submitted,

Robert F. McConnell

ROBERT F. McCONNELL, D.V.M.

181/95

BIOTEK, INC.
STUDY #2172A

MICROSCOPIC FINDINGS

TABLE 1

ANIMAL NO.	105	106	107	108	109
TISSUE/RESPONSE					
HEART	N	N	N	N	N
SPLEEN					
Lymphoid depletion	3	2	1	1	1
Follicular hypoplasia	3	2	1	1	1
Hemosiderin pigment deposits	2	1	3	-	1
LIVER			N	N	
Random hepatocyte vacuolation - lipidosis	1	1	-	-	-
Increased glycogen deposition hepatocytes	-	-	-	-	3
KIDNEY	N	N	N	N	N
URINARY BLADDER	N	N	N	N	N
LYMPH NODE	N	N	N	N	N

LEGEND FOR TABLE
N = tissue within normal histological limits
1, 2, 3, 4 = degree of severity of indicated change
1 = minimal, 2 = mild, 3 = moderate, 4 = marked
- = indicated change not present

Received 2/8/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Awards. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

Encl
as

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management